

Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation technologies: from low level neurophysiological effects to the rehabilitation of stroke patients

Xavier Corominas Teruel

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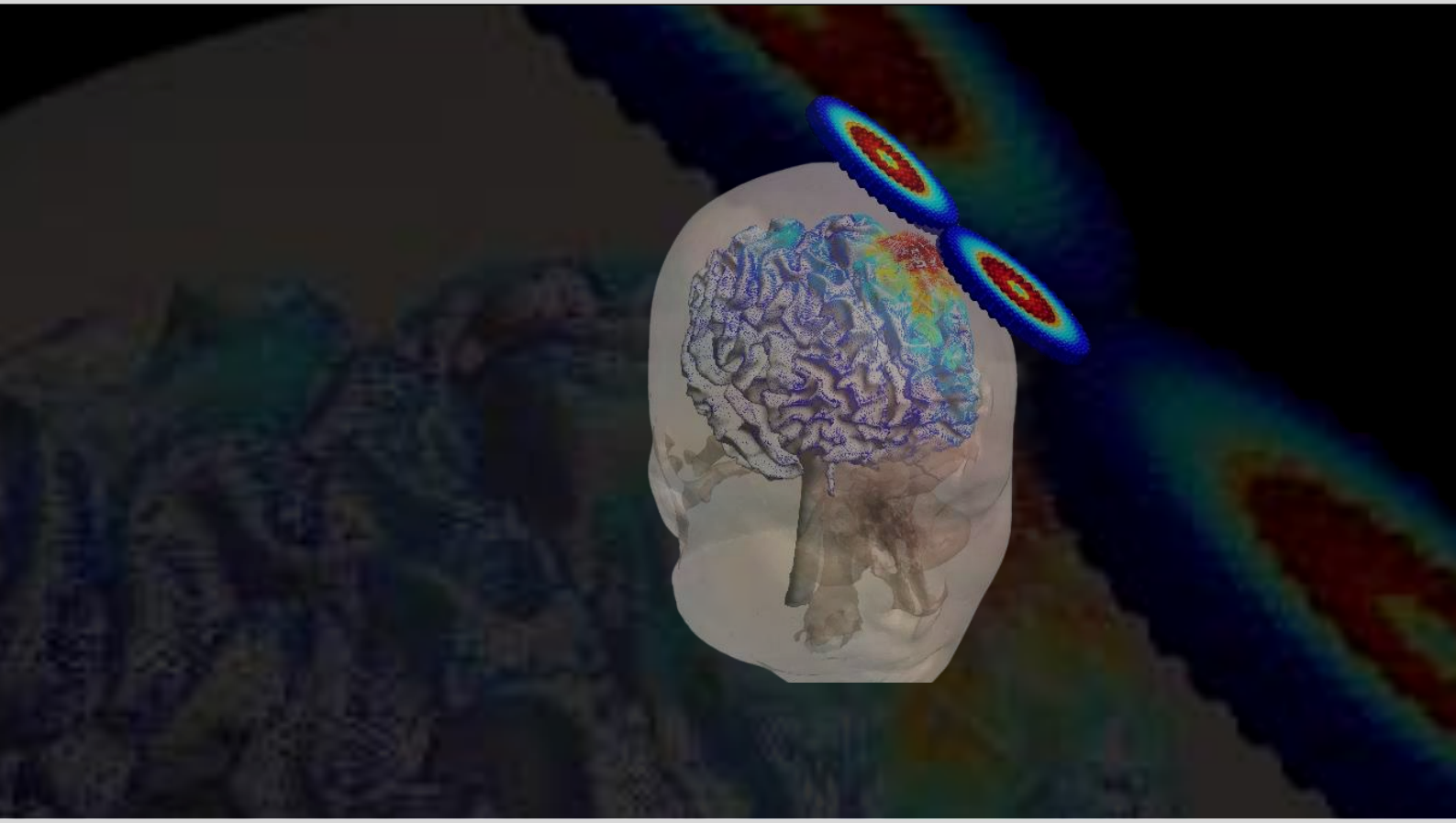
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DOCTORAL DISSERTATION – 2024/25

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This is merely the beginning.

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FAIG CONSTAR que aquest treball, titulat "**Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation technologies: from low level neurophysiological effects to the rehabilitation of stroke patients**", que presenta **Xavier COROMINAS TERUEL** per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament . **Salut, Psicologia y Psiquiatria** d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "**Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation technologies: from low level neurophysiological effects to the rehabilitation of stroke patients**", que presenta **Xavier COROMINAS TERUEL** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento **Salut, Psicología y Psiquiatría** de esta universidad.

I STATE that the present study, entitled "**Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation technologies: from low level neurophysiological effects to the rehabilitation of stroke patients**" presented by **Xavier COROMINAS TERUEL** for the award of the degree of Doctor, has been carried out under my supervision at the Department **Health, Psychology and Psychiatry** of this university.

Tarragona 27 d'octubre de 2024

Els director/s de la tesi doctoral
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SUMMARY

Several decades of brain oscillatory research have provided extensive evidence of their key role in diverse physiological processes at all levels of biological resolution, from the orchestration of molecular release to high-order cognitive functions. Subsequently, a large variety of neurological cognitive and motor disorders arise from dysfunctional spatiotemporal communication of local and distant brain structures, impacting neural and brain function at all levels. Non-invasive brain stimulation (NIBS), especially transcranial electrical stimulation (tES) and transcranial magnetic stimulation (TMS) emerged as translational tools in neurosciences allowing the causal exploration of neural region/network activity with subsequent cognitive processes. Moreover, it also served for diagnostic purposes by probing neuronal excitability levels and allowed game-changing therapeutic applications by driving long-lasting changes in the central nervous system engaging activity-driven mechanisms adaptive for the recovery of damaged brain circuits. However, large inter-individual differences affecting the organization of the human brain, an incomplete understanding of the primary effects of NIBS on neural systems, and an incomplete mapping of the detailed brain circuits involved in neurological symptoms often led to substantial dose-response inconsistencies.

The present PhD dissertation is motivated by a need to develop novel effective stimulation solutions useful to basic and clinically applied research that pave the way for the detection of sensitive biomarkers with adequate spatial resolution and the implementation of novel approaches for effective circuit-based brain therapeutics. The studies framed in this thesis are presented along 3 experimental sections, each one focused on different scientific challenges and employing diverse NIBS and computational tools and methodologies. The first experimental section aims to explore sources of variability of NIBS outcomes and at developing/implementing novel brain-imaging methodologies for improved spatiotemporal electrophysiological signal estimation of the primarily targeted structures in NIBS experiments/interventions. In that experimental section, electroencephalographic (EEG) fundamentals are reviewed and spatial filtering methods derived from electric-field biophysical projections are developed. The second experimental section aims to better understand the primary effects of transcranial magnetic stimulation when delivered at very weak intensities. Throughout a series of experiments combining neuronavigated TMS-EEG-EMG (electromyography), this thesis contributes to novel evidence on the principles of action of TMS which could contribute to explain the interindividual variability encountered in previous studies, and most importantly, pave the way for novel therapeutic neuromodulation strategies to target specifically inhibitory brain disorders. The third and last experimental section aims at implementing and exploring the effects of network-based stimulation methods, with multifocal transcranial direct current stimulation (tDCS) on post-stroke clinical motor and cognitive disorders. Placing stroke patients at the core of an experimental clinical trial employing, this time, multichannel transcranial direct current stimulation (tDCS) coupled to EEG, we tested a novel network-based NIBS protocol targeting sustained attention, motivation and reward and motor learning and adaptation systems in prefrontal and cerebellar regions. Essential parts of the introduction and methodological work here presented has either been published throughout the PhD or is currently under review or preparation. Most importantly the experimental sections are based on actual publications, two of which are accepted and out and five presented as manuscript pre-prints.

The *Introduction* of this PhD dissertation summarizes the latest concepts and findings regarding the human brain functional organization, as well as the role played by intrinsic oscillatory brain activity, neuronal coupling and network synchrony in basic physiological and high-order cognitive processes. Complementary, latest discoveries on mesoscopic plastic properties of the healthy and damaged brain systems following stroke, as well as their critical role in neuromodulation strategies, are presented. Following this, 'NIBS' latest features and applications are summarized, with special attention to TMS and tCS techniques and procedures for personalized therapeutics. Importantly, concurrent, neurophysiological and stimulation modalities with a special emphasis on TMS-EEG are also reviewed. The introduction is completed with a short section presenting the *Challenges and Aims* pursued in the dissertation, stating the underlying scientific questions inspiring the general line of work and the different studies constituting the experimental sections. The relevance of the work, general and specific objectives and hypotheses, and the specific methodological approaches is also framed. A *General Methods* section discusses the experimental, clinical, computational and analytical strategies employed to address the stated scientific questions. Advantages and limitations are listed and critically addressed.

For each of the 3 experimental sections of the dissertation, a summary encompassing the relevant scientific contributions, the context, general aims and specific methods are introduced and the main outcomes also summarized. Note that experimental sections are a comprehensive summary of the manuscripts we produced. Nonetheless, the reader is encouraged to review both. *Experimental section 1* investigates potential sources of variability in NIBS studies; specifically, the variability of neuromodulation strategies and discusses outcomes in post-stroke voluntary motion disorders, identifying the main challenges later on addressed along the following experimental sections and highlighting the need for personalized therapeutics. Not only do NIBS interventions require personalization, but the characterization of inter-individual variability of brain organization also requires personalized brain imaging approaches, likely fundamental for interpreting longitudinal cohorts and inform personalized stimulation in health and disease. To pursue such an ambitious purpose, we have developed a combined neuroimaging-neurophysiological and computational novel framework to spatially filter TMS-EEG readouts, personalizing signal extraction improving the individual reliability and the spatio-temporal resolution of the recorded signals. In *Experimental section 2*, we experimentally investigate phenomena contributing to variability of TMS primary effects. Indeed, TMS has been classically indexed by physiological readouts such motor evoked potentials (MEP) or visual evoked potentials (VEP) requiring high-intensity magnetic fields to prove target engagement. However, two decades of rodent research and new human neurophysiological modalities hypothesized, but not fully demonstrated, that very weak magnetic fields possessed a potential for effective human neuromodulation. Therefore, to explore biological effects of very weak magnetic fields in the human brain, we conducted three TMS-EEG experiments. The first explored the direct *online* effects of single pulse and repetitive TMS stimulation on EEG signals. Employing our previously developed (see Experimental section 1) analytical approach for TMS-EEG filtering, we provided evidence that weak magnetic fields modulates brain activity via different mechanisms of action than conventional high-intensity TMS, operating specifically by increasing spontaneous activity of alpha oscillations, related to inhibitory processes, (rather than entraining

frequency-specific rhythms). A second experiment exploring the role of Intensity-dependent TMS-EEG signatures followed and provided first-time evidence of a physiological entrainment input-output model relating intensities with different primary physiological engaged mechanisms. Last but not least, we conducted a third experiment exploring pre-post repetitive TMS (rTMS) offline cortical excitability signatures, demonstrating post-rTMS modulation of the human primary motor cortex, suggesting a potential for neuromodulation for weak-intensity TMS and an inhibitory nature. Finally, *Experimental section 3* was instigated by the need to develop stimulation protocols based on inter-regional network communication. Robust multimodal evidence has demonstrated that brain networks are causally correlated and anticorrelated (i.e., temporally synchronized) exchanging information and driving functional reorganization episodically or by waves. However, most neuromodulation interventions aimed at the rehabilitation of neurological deficits lack a network perspective. We here developed, implemented and explored the potential effects of tDCS modulation aiming at extended networks in post-stroke outcomes following a middle cerebral artery strokes. More specifically, we developed a targeting tDCS strategy based on increasing cortical activity in distant regions potentially affected by diaschisis effects. Even if partially inconclusive and in need of larger sample recruitment, clinical outcomes highlight the clinical potential of the new approach in stroke and the need for future personalized strategies conditioned by baseline lesion and interindividual brain-state features.

Altogether, we first demonstrate that a custom-developed TMS-EEG filtering analytical strategy attests a high potential in individually decoding neural signatures in the primarily targeted neural assemblies, incrementing spatial specificity of the recorded signals and setting the stage for future brain-state dependent technological advances in the field of stimulation. Next, we provide evidence that weak magnetic fields could induce increases of spontaneous activity and neuronal entrainment, potentially with a predominant oscillatory inhibitory effect on the human brain. These findings could contribute to explain variability of TMS physiological effects as well as contribute, for the first time, to the development of a system-specific neurotherapeutic target to selectively manipulate the activity of inhibitory systems. Finally, we show that multifocal stimulation can be implemented safely in clinical settings, and healthy participants or patients might directly benefit from more sophisticated novel approaches aiming to manipulate networks rather than isolated cortical regions. More generally, the studies here presented address scientific and technical challenges posed by different NIBS allow us to *conclude* that personalized brain-informed network-wide specific neuromodulation is feasible and might set the stage for future research in brain stimulation aiming to manipulate networked brain oscillations and synchrony in basic and clinical research applications.

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RESUMEN (ESPAÑOL)

Varias décadas de investigación en oscilaciones cerebrales han proporcionado amplia evidencia de su papel clave en diversos procesos fisiológicos a todos los niveles de resolución biológica, desde la orquestación de la liberación de neurotransmisores hasta las funciones cognitivas de alto orden. Consecuentemente, una gran variedad de trastornos neurológicos, cognitivos y motores surgen de la comunicación espaciotemporal disfuncional entre estructuras cerebrales locales y distantes, afectando la función neuronal y cerebral. La estimulación cerebral no invasiva (NIBS, por sus siglas en inglés), especialmente la estimulación eléctrica transcraneal (tES) y la estimulación magnética transcraneal (TMS), emergieron como una familia de herramientas traslacionales que permiten en el campo de las neurociencias la exploración causal de la actividad de regiones/redes relacionadas con procesos cognitivos. Además, también sirven amplios fines diagnósticos al evaluar los niveles de excitabilidad neuronal y permitiendo aplicaciones terapéuticas revolucionarias al inducir cambios duraderos en el sistema nervioso central, dirigiendo mecanismos actividad-dependientes que resultan adaptativos para la recuperación de circuitos cerebrales dañados. Sin embargo, las grandes diferencias interindividuales en la organización del cerebro humano, la incompleta comprensión de los efectos primarios de la NIBS sobre las mismas y el mapeo parcial de circuitos cerebrales involucrados en síntomas neurológicos específicos conducen a incongruencias sustanciales de la relación dosis-respuesta.

La presente tesis está motivada por la necesidad de implementar nuevas soluciones de estimulación efectivas para la investigación básica y clínica, allanando el camino para la detección de biomarcadores fisiológicos sensibles con una resolución espacial sin precedentes e implementando nuevos enfoques para terapias cerebrales basadas en circuitos. Los estudios enmarcados en esta tesis se presentan a lo largo de 3 secciones experimentales, cada una enfocada en diferentes desafíos científicos y empleando diversas metodologías de NIBS y computacionales. La primera sección experimental tiene como objetivo explorar las fuentes de variabilidad de los resultados de NIBS y desarrollar e implementar nuevas estrategias de neuroimagen y neurofisiología para mejorar la sensibilidad espaciotemporal en los registros electrofisiológicos de las estructuras principalmente estimuladas en los experimentos/intervenciones con NIBS. En esta sección, se revisan los fundamentos de la electroencefalografía (EEG) y se desarrollan métodos de filtrado espacial derivados de proyecciones biofísicas del campo eléctrico. La segunda sección experimental tiene como objetivo comprender mejor los efectos primarios de la estimulación magnética transcraneal. A través de una serie de experimentos que combinan TMS-EEG-EMG y neuronavegacion, esta tesis contribuye nuevas evidencias sobre los principios de acción de la TMS que podrían en parte explicar la variabilidad de la respuesta encontrada en estudios previos, y lo más importante, allana el camino para la implementación de nuevas estrategias terapéuticas de neuromodulación dirigidas específicamente a trastornos cerebrales inhibitorios. La tercera y última sección experimental tiene como objetivo diseñar y explorar los efectos de los métodos de estimulación basados en redes, con estimulación transcraneal de corriente directa multifocal (tDCS) en trastornos clínicos de movimiento y cognitivos post-ictus. Situando al paciente en el centro de la investigación, enmarcando la tDCS en un ensayo experimental clínico, se desarrolla e implementa un protocolo de NIBS basado en circuitos que tiene como objetivo las redes prefrontales y cerebelosas dedicadas a la atención sostenida/motivación y recompensa,

la planificación, reaprendizaje y readaptación motoras. La parte esencial del trabajo aquí presentado se ha publicado o está pendiente de revisión o preparación a lo largo de este doctorado y las secciones experimentales presentadas en esta tesis se basan en las publicaciones, dos de las cuales están publicadas al momento de escribir y cinco están integradas presentadas como manuscritos pre-impresos (*preprints*) adjuntos.

La *Introducción* de esta tesis resume los conceptos y hallazgos más recientes sobre la organización funcional y estructural del cerebro humano, así como el papel de la actividad oscilatoria intrínseca del cerebro, el acoplamiento neuronal y la comunicación sincrónica de redes en procesos fisiológicos básicos y cognitivos. De forma complementaria, se presentan los últimos descubrimientos sobre las propiedades plásticas mesoscópicas del cerebro sano y dañado en contextos post-ictus, así como su papel crítico en las estrategias de neuromodulación. A continuación, se resumen las características y aplicaciones más recientes de la estimulación cerebral no invasiva (NIBS), con especial atención a las técnicas y procedimientos de TMS y tCS para terapias personalizadas. Conjuntamente se revisan las modalidades concurrentes de neuroimagen y de estimulación con especial interés en TMS-EEG. La introducción se completa con una breve sección que presenta los *Desafíos y Objetivos* perseguidos, exponiendo las preguntas científicas subyacentes que inspiran la línea general de trabajo y los diferentes estudios que constituyen las secciones experimentales. Se detallan la relevancia del trabajo, los objetivos generales y específicos, las hipótesis y los enfoques metodológicos generales. A continuación, se presenta una sección de Métodos Generales, que discute las metodologías experimentales, clínicas, computacionales y de análisis de datos empleadas para abordar las preguntas científicas. Se detallan y caracterizan críticamente las ventajas y limitaciones de cada uno de ellos.

Para cada uno de ellos, se presenta un resumen de los contenidos en las publicaciones científicas, introduciendo primero los Objetivos Específicos y los Métodos más relevantes, seguidos de los Resultados utilizando cada vez un formato de artículo científico. Las secciones experimentales contienen un resumen de las principales contribuciones científicas de cada estudio, por lo que se recomienda al lector a revisar tanto el resumen como los Manuscritos en si mismos ya que contienen información complementaria. En primer lugar, la sección experimental 1 investiga las posibles fuentes de variabilidad en los estudios de NIBS; específicamente, se discuten la variabilidad de las estrategias de neuromodulación y los resultados en los trastornos del movimiento post-ictus, destacando la necesidad de terapias personalizadas. Además, se argumenta que las intervenciones de NIBS no solo requieren personalización en su aplicación, sino que también es necesario abordar el estudio de la variabilidad interindividual de la organización cerebral mediante procedimientos de neuroimagen/neurofisiología cerebral personalizados, que pueden ser fundamentales para interpretar cohortes longitudinales e informar sobre la estimulación personalizada, tanto en cohortes sanas como en presencia de patología. Para dicho propósito, se desarrolla un nuevo marco de métodos de neuroimagen, neurofisiología y computacionales para filtrar espacialmente las señales de TMS-EEG, personalizando la extracción de señales y mejorando la fidelidad individual así como la resolución espaciotemporal de los registros. A continuación, en la sección experimental 2, se investigan experimentalmente fenómenos poco explorados que subyacen a los efectos primarios de TMS y que contribuyen potencialmente a la variabilidad observada en la

literatura. La TMS se ha indexado clásicamente mediante lecturas fisiológicas como potenciales evocados motores (MEP) o potenciales evocados visuales (VEP) que requieren campos magnéticos de alta intensidad para demostrar la presencia de actividad. Sin embargo, dos décadas de investigación en roedores y nuevas modalidades de neurofisiología han planteado la hipótesis -aún no demostrada- que campos magnéticos muy débiles en intensidad ('weak or *very weak TMS'*) también podrían mostrar potencial de neuromodulación en humanos, contribuyendo así a explicar décadas de variabilidad fisiológica posterior de TMS convencional. Para explorar los efectos neurofisiológicos de dichos campos magnéticos en el cerebro humano, se plantearon experimentos combinando TMS-EEG. El primer experimento explora los efectos directos "online" de la estimulación con pulsos únicos y repetitivos. Empleando nuestros nuevos métodos previamente desarrollados (en la sección experimental 1) para el filtrado de señales TMS-EEG, proporcionamos evidencia de que los campos magnéticos débiles podrían operar mediate diferentes mecanismos fisiológicos de acción que la TMS convencional a alta intensidad, aumentando principalmente la actividad espontánea de oscilaciones alfa, clásicamente relacionadas con efectos neuronales inhibitorios, en lugar de imponer vía '*entrainment'* ritmos de frecuencia específica. Un segundo experimento explora los correlatos de TMS-EEG dependientes de la intensidad. A pesar de las limitaciones de los artefactos al comparar señales de EEG a distintos niveles de intensidad de estimulación por TMS, presentamos evidencia de un modelo fisiológico entrada-salida (input-output) que relaciona las intensidades con diferentes mecanismos fisiológicos primarios facilitados. Finalmente, un tercer y último experimento explora los índices de excitabilidad cortical pre-post TMS repetitiva (rTMS) en un modelo "offline", demostrando una inhibición sostenida generada por TMS repetitiva (rTMS) a baja intensidad de la corteza motora primaria humana, sugiriendo su naturaleza inhibitoria y su potencial de neuromodulación. Finalmente, la sección experimental 3, aborda la necesidad de desarrollar protocolos de estimulación basados en la modulación de la comunicación en redes interregionales. Evidencias multimodales robustas han mostrado que la actividad en el seno de redes cerebrales extensas puede estar correlacionada (sincronizada temporalmente) o anticorrelacionada, utilizando dicho mecanismo para intercambiar información y conduciendo a la reorganización funcional en episodios de daño cerebral. Sin embargo, la mayoría de las intervenciones de neuromodulación aborda los déficits neurológicos sin considerar dicha perspectiva de red. En esta sección, se exploran los posibles efectos de modulación terapéutica de la tDCS muticanal en modalidad multi-red sobre las capacidades cognitivas y motoras después de un accidente vascular cerebral en el territorio de la arteria cerebral media. Nuestros resultados destacan el potencial del nuevo enfoque y la necesidad de futuras estrategias personalizadas, condicionadas por las características de la lesión.

En conjunto, concluimos que nuestro enfoque metodológico de filtrado de señales TMS-EEG muestra un buen potencial para la codificación individual de efectos sobre las áreas primariamente estimuladas, incrementando la especificidad espacial de las señales registradas, estableciendo así las bases para futuros avances tecnológicos. A continuación, concluimos que los campos magnéticos débiles podrían tener un efecto predominantemente inhibitorio en el cerebro humano. Dichos hallazgos contribuyen a explicar en parte la variabilidad de los efectos neurofisiológicos de la TMS y permiten proponer, por primera vez, como objetivo neuroterapéutico la manipulación selectiva la

de sistemas inhibitorios. Finalmente, demostramos que la estimulación multifocal se puede implementar clínicamente de manera segura y que los pacientes pueden beneficiarse directamente de nuevos enfoques que buscan manipular redes cerebrales enteras en lugar de regiones corticales individuales. De manera más general, los estudios aquí presentados abordan desafíos científicos y técnicos en diferentes frentes abiertos del campo de las NIBS, *concluyendo* que la neuromodulación personalizada informada por las características estructurales del cerebro, especialmente de sistemas inhibitorios y organizados en red es factible, y podría permitir un aumento de la efectividad de las intervenciones de estimulación destinadas a manipular la sincronía y las oscilaciones cerebrales en aplicaciones de investigación fundamental y clínica.

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RESUM (CATALÀ)

Diverses dècades de recerca sobre el rol de les oscil·lacions cerebrals han proporcionat evidència del seu paper clau en múltiples processos fisiològics a diferents nivells de resolució biològica, des de l'orquestració de l'alliberament de neurotransmissors fins a funcions cognitives d'alt nivell. Conseqüentment, una gran varietat de trastorns neurològics, cognitius i motors sorgeixen de disfuncions en la comunicació espai-temporal entre estructures cerebrals locals i distants, afectant la funció cerebral. L'estimulació cerebral no invasiva (NIBS, segons les seves sigles en anglès), i molt especialment l'estimulació elèctrica transcranial (tES) i l'estimulació magnètica transcranial (TMS), van emergir com una família d'eines translacionals que permeten, en el camp de les neurociències, l'exploració causal de l'activitat de regions o xarxes relacionades amb processos cognitius. A més, també tenen aplicacions diagnòstiques en l'avaluació dels nivells d'excitabilitat neural, fent possible així aplicacions terapèutiques revolucionàries i canvis a llarg termini en l'activitat del sistema nerviós central, mitjançant mecanismes dependents d'activitat potencialment adaptatius per a la recuperació de circuits cerebrals afectats. No obstant això, notables diferències interindividuals en l'organització del cervell humà, una comprensió parcial dels efectes de la NIBS i una cartografia incompleta dels circuits cerebrals implicats en símptomes neurològics específics han conduït a incongruències substancials dels efectes dosi-resposta.

Aquesta tesi doctoral està motivada per la necessitat d'implementar noves solucions d'estimulació efectives per a la recerca bàsica i aplicada clínica, que facilitin el camí cap a la detecció de biomarcadors fisiològics sensibles amb una adequada resolució espacial i nous enfocaments de teràpies de neuromodulació cerebral basada en la manipulació de xarxes. Els estudis emmarcats en aquesta tesi es presenten en 3 seccions experimentals, cadascun enfocat en diferents desafiaments científics i utilitzant diverses metodologies de NIBS i computacionals. La primera secció experimental té com a objectiu explorar les fonts de variabilitat dels efectes de NIBS i desenvolupar i implementar noves metodologies de neuroimatge per a millorar la sensibilitat espacial i temporal dels registres electrofisiològics de les estructures principalment estimulades en els experiments/intervencions amb NIBS. En aquesta secció, es revisen els fonaments de l'electroencefalografia (EEG) i es desenvolupen mètodes de filtratge espacial derivats de projeccions biofísiques del camp elèctric sobre la superfície cortical. La segona secció experimental té com a objectiu comprendre millor els efectes primaris de l'estimulació magnètica transcranial. A través d'una sèrie d'experiments que combinen TMS-EEG-EMG i neuronavegació, aquesta tesi contribueix nova evidència al coneixement existent sobre els principis d'acció de la TMS que podrien explicar parcialment la variabilitat interindividual trobada en estudis previs de TMS, i aplana el camí per a desenvolupar noves estratègies terapèutiques de neuromodulació de tipus inhibitòria dirigides específicament a tractar trastorns cerebrals.

La tercera i darrera secció experimental té com a objectiu implementar i explorar els efectes de mètodes d'estimulació sobre àmplies xarxes corticals, amb estimulació transcranial de corrent directe (tDCS) multifocal/multicanal en trastorns clínics motors i cognitius post-ictus. Situant un grup de pacients afectats per accidents cerebrovasculars al cor del projecte i un règim d'estimulació per tDCS en el context d'un assaig clínic experimental, s'implementa un protocol de NIBS basat en la modulació de circuits amb

l'objectiu de modular xarxes prefrontals i cerebel·loses implicades en l'atenció sostinguda, la motivació i la recompensa, així com l'aprenentatge i la readaptació motores. Una part essencial del treball que aquí es presenta ja ha estat publicada o es troba actualment en vies de revisió o preparació, mentre que les seccions experimentals que formen part d'aquesta memòria de tesi doctoral es basen en el contingut de dos articles ja publicats i cinc altres que són inclosos com a manuscrits prepublicats (preprints).

L'*Introducció* d'aquesta tesi resumeix els conceptes i troballes més recents sobre l'organització estructural i funcional del cervell humà, així com el paper de l'activitat oscil·latòria cerebral intrínseca, el *coupling* neural i la sincronia d'activitat i la comunicació en xarxes en processos fisiològics i cognitius. De forma complementària, presentem descobriments recents sobre les propietats plàstiques del cervell sa i danyat post-accident vascular cerebral des d'una perspectiva mesoscòpica, explicant-ne el seu paper crític per a desenvolupar estratègies eficaces de neuromodulació. A continuació, es resumeixen les característiques i les aplicacions més recents de l'estimulació cerebral no invasiva (NIBS), amb especial atenció a les tècniques i procediments de TMS i tES com a teràpies personalitzades. També es revisen les modalitats concurrents de neuroimatge/neurofisiologia i estimulació cerebral, posant l'accent en la combinació TMS i EEG. La introducció es completa amb una breu secció que presenta els Desafiaments i Objectius abordats en aquesta memòria de tesi, incloent-hi les qüestions científiques subjacents que inspiren les línies de treball i els diferents estudis que constitueixen cada secció. S'accentuen la rellevància del treball, els objectius generals i específics que l'inspiren i les hipòtesis que se'n deriven, així com les estratègies metodològiques generals. Una secció de Mètodes Generals discuteix les metodologies experimentals, clíniques i computacionals, així com les estratègies d'anàlisi de dades utilitzades, detallant per a cadascun d'aquests aspectes els avantatges i limitacions.

El objectius de la tesi s'aborden en tres seccions, per cadascuna dels quals es presenta un resum de l'estat de la qüestió recolzat en publicacions científiques, detallant els seus *Objectius Específics* i els *Mètodes* més rellevants, seguits dels seus *Resultats,* utilitzant cada vegada un format d'article científic. Les seccions contenen només un resum de les principals contribucions científiques de cada estudi, raó per la qual es recomana al lector la lectura completa dels *Manuscrits* amb informació detallada i complementària.

La secció experimental 1 investiga les possibles fonts de variabilitat en estudis de NIBS. Específicament, es discuteix la variabilitat de les estratègies de neuromodulació i els seus resultats en trastorns del moviment post-ictus, posant l'accent sobre la necessitat de teràpies personalitzades. A més, s'argumenta que les intervencions de NIBS requereixen no només una individualització en la seva aplicació sinó de tenir en compte la variabilitat interindividual de l'organització cerebral mitjançant procediments de neuroimatge cerebral, fonamentals per a interpretar estudis de cohorts i guiar els procediments d'estimulació personalitzada. Amb aquesta finalitat, es disenya i desenvolupa un nou procediment computacional que permet filtrar els senyals de TMS-EEG, personalitzant l'anàlisi d'activitat EEG pertinent i millorant la resolució espacial i temporal dels registres d'EEG i la consistència individual dels anàlisis. La secció experimental 2, investiga experimentalment fenòmens poc explorats subjacents als efectes primaris de la TMS i que contribueixen a la variabilitat de la resposta observada

en la literatura. Els efectes de la TMS s'han indexat clàssicament per la presència de potencials evocats motors (MEP) o potencials evocats visuals (VEP) induits per impulsions magnètiques d'alt voltatge. No obstant això, dues dècades de recerca en rossegadors i noves tècniques de neuroimatge han permès de plantejar la hipòtesi -encara no demostrada- que polsos magnètics febles o molt febles (*weak* or *very weak magnetic fields)* poden generar efectes de neuromodulació en humans. En aquest context, i per posar en evidència els efectes cerebrals de camps magnètics molt febles amb TMS-EEG, un primer experiment que explora els efectes "*online*" (simultanis) mitjançant estimulació amb polsos únics i/o repetitius. Emprant mètodes prèviament desenvolupats (secció 1) per filtrar espacialment senyals de TMS-EEG mostrem que els camps magnètics febles actuarien a través de diferents mecanismes d'acció que els efectes de la TMS convencional d'alta intensitat, amb un increment de l'activitat espontània d'oscil·lacions alfa, relacionades clàssicament amb efectes inhibitoris, en lloc d'imposar per *entrainment* ritmes específics a la freqüència alliberada. Un segon experiment explora els efectes de la intensitat d'estimulació sobre senyals TMS-EEG. Malgrat les limitacions causades pels artefactes electromagnètics dels polsos sobre els registres d'EEG, els resultats d'aquest estudi mostren un model fisiològic d'entrada-sortida (*input-output*) que recolza la hipòtesi de diferents mecanismes de facilitació. Finalment, un tercer experiment explora canvis del nivell d'excitabilitat cortical "*offline*" en resposta a la TMS repetitiva (rTMS), mostrant efectes d'inhibició de l'escorça motora primària humana, i per tant el potencial de neuromodulació de carácter inhibitori i de TMS a baixa intensitat. En la sección experimental 3, s'aborda la necessitat de desenvolupar protocols d'estimulació terapèutica dirigits a la modulació de xarxes cortico-corticals. Evidència sòlida demostrant que les xarxes cerebrals oscil·len entre estats de correlació (sincronització temporal) i anticorrelació i en funció dels mateixos faciliten l'intercanvi d'informació i afavoreixen la reorganització funcional després d'episodis de dany cerebral. No obstant, la majoria de les intervencions de neuromodulació actuals no aborden el tractament dels dèficits neurològics amb una perspectiva de xarxa. En aquesta tesi implementem i evaluem els efectes de la modulació mitjançant un règim de tDCS multicanal d'una xarxa de regions prefrontals i cerebel·loses sobre la recuperació motora i cognitiva després d'un accident vascular cerebral que afecta el territori de l'artèria cerebral mitjana. Els nostres resultats indiquen el potencial d'aquest enfoc i emfatitzen la necessitat de futures estratègies de neuromodulació personalitzada, condicionades per les característiques basals de la lesió.

En conjunt, els resultats d'aquesta tesi permeten concloure en primer lloc que la nostra tècnica de filtratge de senyals TMS-EEG mostra un elevat potencial per a la descodificació individual de signatures neuronals de les àrees primàriament estimulades, incrementant l'especificitat espacial dels senyals registrats i establint les bases per a futurs avanços tecnològics. En segon lloc, concloem que els patrons d'impulsions magnètiques a feble intensitat podrien tenir un efecte de modulació cortical, predominantment inhibitori. Aquesta troballa podria contribuir a explicar una part de la variabilitat dels efectes fisiològics observats per a la TMS així com permetria, per primera vegada, establir una nova estratègia neuroterapéutica centrada en manipular selectivament l'activitat de sistemas corticals inhibitoris. Finalment, els nostres resultats permeten mostrar que l'estimulació tDCS multifocal es pot implementar clínicament de manera segura i que els pacients podrien beneficiar-se d'enfocaments dirigits a modular

xarxes cerebrals en lloc de regions corticals individuals. De manera mes general, els resultats d'aquesta tesi atorguen respostes i sol·lucions a diferents desafiaments científics i tècnics del camp de la NIBS, i permeten *concloure* que la neuromodulació personalitzada informada per les característiques estructurals, especialment de sistemas inhibitoris i organitzats en xarxa és factible, i podria augmentar l'efectivitat de les intervencions d'estimulació cerebral en recerca fonamental i clínica, mitjançant la modulació de la sincronia i les oscil·lacions cerebrals.

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LIST OF ABBREVIATIONS

cTBS: Continuous Theta Burst Stimulation

EEG: Electroencephalography

E-field: Electric Field

EMG: Electromyography

ERP: Event Related Potential

ICA: Independent Component Analyses

iTBS: Intermittent Theta Burst

ITPC: Intertrial Phase Coherence

MEP: Motor Evoked Potential

MNE: Minimum Norm Estimator

MRI: Magnetic Resonance Imaging

MSO: Maximum Stimulator Output

MT: Motor Threshold

M1: Primary Motor Cortex

NIBS: Non-invasive Brain Stimulation

PCA: Principal Component Analyses

RMT: Resting Motor Threshold

rMT: Resting Motor Threshold

rTMS: Repetitive Transcranial Magnetic Stimulation

EP: Evoked Potentials

tACS: Transcranial Alternating Current Stimulation

TCS: Transcranial Current Stimulation

tDCS: Transcranial Direct Current Stimulation

TES: Transcranial Electrical Stimulation

TMS: Transcranial Magnetic Stimulation

TMS-EEG: Concurrent TMS-EEG (Electroencephalography)

tRNS: Transcranial Random Noise Stimulation

VEP: Visual Evoked Potentials

LIST OF PUBLICATIONS

This doctoral dissertation gathers the following experimental reports which are further referred to in the text by their assigned numbers. For a better comprehension and fluency of the dissertation, the manuscripts associated with experimental sections of the dissertation can be found as part of their corresponding experimental sections.

1. **Corominas-Teruel, X**., Mozo, R. M. S. S., Simó, M. F., Colomina Fosch, M. T., & Valero-Cabré, A. (2022). Transcranial direct current stimulation for gait recovery following stroke: A systematic review of current literature and beyond. Frontiers in Neurology, 13. https://www.frontiersin.org/articles/10.3389/fneur.2022.953939.
2. **Corominas-Teruel, X** et al. (2024). E-field-based spatial filtering for a more accurate estimation of locally EEG responses evoked with Transcranial Magnetic Stimulation. Manuscript preprint.
3. **Corominas-Teruel, X** et al. (2024). Oscillatory biomarkers of Transcranial Magnetic Stimulation impact at very weak-intensity: a TMS-EEG study. Manuscript preprint.
4. **Corominas-Teruel, X** et al. (2024). Intensity-dependent oscillatory correlates of Transcranial Magnetic Stimulation in the human brain. Manuscript preprint.
5. **Corominas-Teruel, X**. et al. (2024). Can very weak intensity repetitive Transcranial Magnetic Stimulation modulate cortical excitability? Manuscript preprint.
6. **Corominas-Teruel, X**., Bracco, M., Fibla, M., Segundo, R. M. S., Villalobos-Llaó, M., Gallea, C., Beranger, B., Toba, M., Valero-Cabré, A., & Colomina, M. T. (2023). High-density transcranial direct current stimulation to improve upper limb motor function following stroke: study protocol for a double-blind randomized clinical trial targeting prefrontal and/or cerebellar cognitive contributions to voluntary motion. Trials, 24(1), 783. https://doi.org/10.1186/s13063-023-07680-8.
7. **Corominas-Teruel, X** et al. (2024). Transcranial direct current stimulation on prefrontal and/or cerebellar systems to improve motor function in stroke patients: Preliminary analyses from an ongoing pilot study. Manuscript preprint.

These seven reports summarize the major contributions of the present PhD dissertation at the time it is submitted for evaluation. Four of the reports are presented as *Original Research Manuscripts* (1 systematic review (**m.1**),1 methodological report (**m.6**), 2 experimental reports (**m.3&4**)); Two reports are presented as short manuscript reports (1 technical report(**m.2**), 1 short experimental report(**m.7**)); one final report is presented as a short Letter to the editor (**m.5**). Reports are summarized into the thesis dissertation in their respective experimental sections. Together with the scientific publications, supplementary research material comprising relevant scientific contributions are also listed in adjacent document of activities (SAD) and appended to this dissertation but not in manuscript format.

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1. INTRODUCTION

1.1. Neurophysiology of the human brain

Centuries of neuroscience research have firmly evidenced that the human brain is a complex organ hierarchically organized across multiple levels of biological resolution. From the molecular transmission, cellular organization, synaptic communication, population dynamics, to parcellated areas part of larger systems/networks engaging in a common brain enabling high-order cognitive function, all spatial scales remain causally connected (Figure 1).

The brain is a dynamic organ capable of adapting, reorganize its structural and functional resources and evolve at all biological levels conditioned by the patterns of external and internal inputs[1,2]. This unique property, commonly known as *neural plasticity*, structures the physiological foundation of a long list of medical diagnostic and interventional tools which play a major role in today's healthcare. Importantly, several brain functions and properties, and notably neural plasticity, are largely driven by neuronal activity[3–8]. This specific property featured by the nervous system implies that neural activity is essential to flexibly maintain, weaken or strengthen neuronal connections, information transfer and processing, conveying a potential to regulate bidirectional interactions at any biological scale, hence between the extremes of a gradient between high (cognition) and low level (neurochemical transmission) processes. Additionally, the perturbation or modulation of neuronal activity has traditionally allowed the causal exploration of its mechanisms, enabling neuroscientists investigate the interplay between brain structure and function.

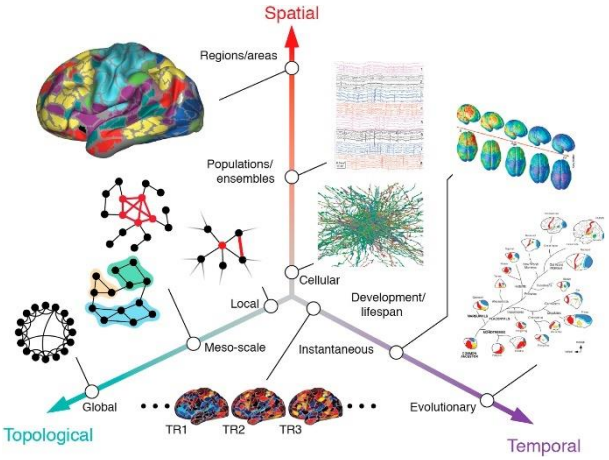


Figure 1. Brain organization. Illustration of multiscale hierarchical organization of the human brain (Adapted from[2])

Since the mid 19th century scientists have attempted to regionalize the structure of the brain to then study its function, making of areal and circuits delineation one of the key achievements of systems neuroscience[9]. Historically, pathological brain lesions provided the foundations to associate structure and function, typifying damage-induced deficits according to the localization of the impacted brain regions**.** Moreover, therapeutic lesions were also leveraged as primary interventions for neurological and psychiatric disorders. For decades, serendipitous findings guided the exploration of human brain structure and function associations and the development of therapeutic implications[10]. However, by the 1980s, reliance on lesion-studies declined due to the development of magnetic resonance imaging (MRI), positron emission tomography (PET) and particularly functional MRI (fMRI). These technologies allowed the exploration of the human brain at, at the time, unprecedented levels of resolution in larger cohorts, depending on individual brain cases and serendipitous findings (Figure 2).

However, despite obvious ethical concerns and limitations, lesion studies had one remarkable advantage over functional imaging studies; they enabled the establishment of causal links between affected brain regions and associated symptoms hence opening a window into their functions in healthy condition. Fortunately, lesions and neuroimaging studies did not mutually exclude each other and provided complementary information and kept making progress together. Lesions offered initial evidence on the putative role of specific brain structures (and its associated networks) in specific functions, while neuroimaging was more suited to reveal the spatiotemporal activity patterns of such contributions. More recently, from the mid 90' to the present day, the detailed dissection of the brain into contiguous parcelled regions integrating architecture, connectivity and function has been facilitated by the combination of datasets provided by causal tools with the ability to perturb neural function, such as electroconvulsive therapy (ECT)[11], transcranial electrical stimulation (tES)[12], transcranial magnetic stimulation (TMS)[13], and deep brain stimulation (DBS)[14], along increasingly spatially precise neuroimaging modalities such 3T/7T MRI, intracranial EEG recordings (SEEG) and high density electroencephalography (EEG) associated to sophisticated analytical methods lesion-symptom mapping[15,16] or deep learning/neural networks.

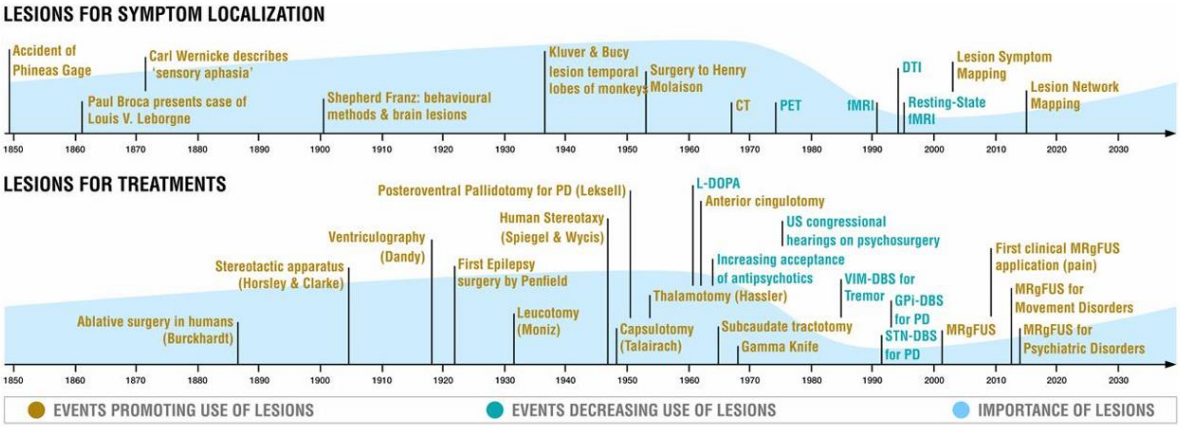


Figure 2. Causal neuroimaging historical events. Historical timeline illustrating the role of lesions in brain structure-to-function causal explorations and the development of non-reversible brain lesion clinical interventions. Blue background continuous line represents the frequency of lesion use embedding historical events temporally related to the rise, fall, and return of lesions (Adapted from[10]).

These techniques have been proven instrumental in advancing the spatial localization of brain processes on specific structures and areas, but most importantly, they are paramount for characterizing how different regions interact within the context of local and long-range systems leading to the engagement of more complex high level functions. It is on such methodological background that, **over the past decade the neuroscience community has witnessed a paradigm shift, stablishing neuronal-to-neuronal communication (i.e., synapses) as the primary computational unit of the brain, rather than the neuronal structural cells themselves**[8], highlighting the uniqueness of the brain as a activity-dependent system.

1.1.1 Recording brain neural dynamics: Organization of brain resources in health

The early convergence of causal (real lesions and reversible perturbations) and correlational (neuroimaging and neurophysiological) approaches, allowed the scientific community to explore the spatiotemporal properties of local and inter-regional neural communication (networks) and their role in human brain function in both health and disease. This endeavour, through multimodal invasive and non-invasive recordings (Figure 3), has established that temporally and frequency precise local and network neuronal synchronization/de-synchronization is critical for coherent cognition and behaviour[17]. Moreover, robust evidence has shown cross-network interactions balance functionally correlated and anticorrelated activity based on external environmental stimuli. **However, how do brain systems coordinate this reciprocal mosaic of activity to produce coherent cognitive and behavioural outputs?.** Multiple research domains within the neurosciences have converged on the notion that local and highly distributed coordinated neural transient activity poses a comprehensive framework to address this question[18–20].

Within this framework, patterns of transient activity operating at the millisecond scale can be recorded using electrophysiological measurements. From the local single-unit scale (single neurons) to the large-scale level (neural assemblies forming more complex interconnected networks), neurophysiological approaches offer the opportunity to study brain activity at the highest levels of temporal resolution. Decades of research have shown that neuronal structures in primary sensory areas, for example, are cytoarchitecturally organized, clustered into cortical columns composed of excitatory and inhibitory populations that

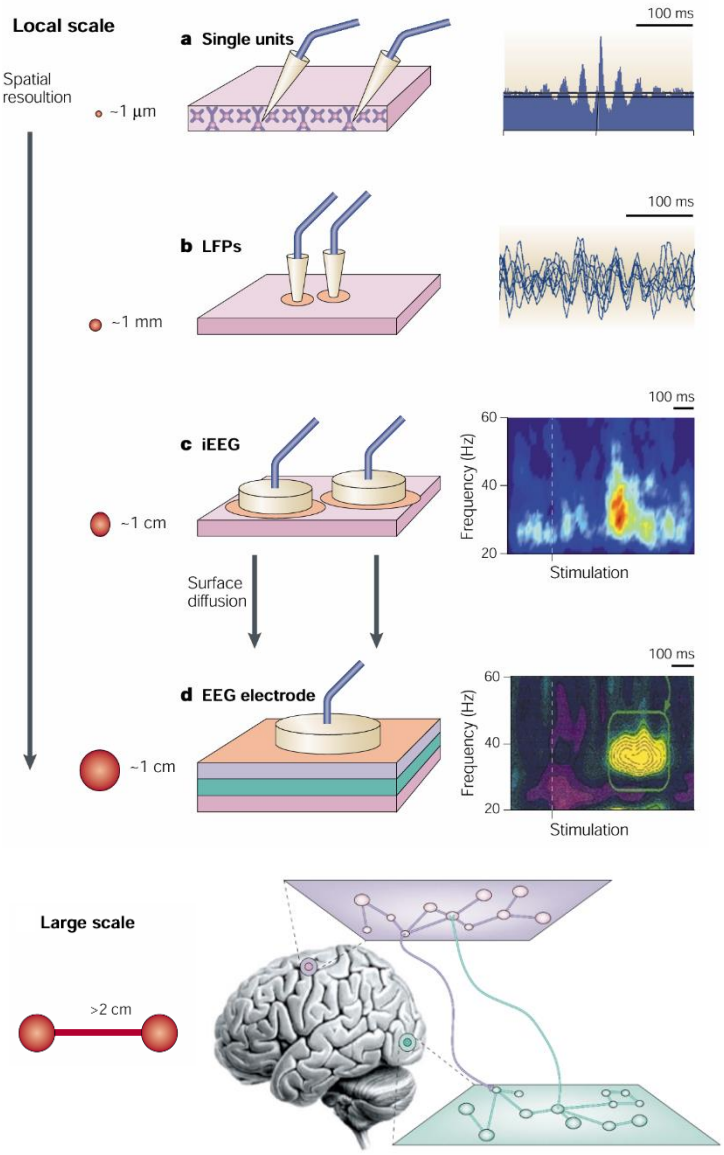


Figure 3. Recording human brain activity. Illustration of distributed neural assemblies engaging in both local and long-range communication. Different electrophysiological techniques are required to capture the diversity of neural dynamics, with each contributing information about electrical polarization/depolarization processes at varying levels of spatial resolution (Adapted from[18]).

synchronize into common local resonant oscillatory rhythms[18]. Moreover, animal and human research has largely shown that single neural activity is highly dependent on local synchronization at the mesoscopic level, both determined by local structural connectivity features[18]. Likewise, evidence generated separately by local field potentials (LFP) and intracranial EEG measurements (iEEG, also referred to as stereotaxic EEG, SEEG) extracranial electroencephalography (EEG) and magnetoencephalography (MEG), has provided converging evidence pointing at rhythmic oscillations as a representative signature of neural communication and processing[21,22].

Oscillatory rhythms were first well characterized in the early 70s in felines and humans sensory processing, sleep and consciousness states; however considered for many as epiphenomenomena with unclear links, or no relevant links, to cognition or behaviour[23]. Later on, from the mid-90s to the early 2000s oscillations benefited from renewed attention and first examples in non-human primates linked such to physiological phenomena such as communication, processing and the orchestration of high-order cognitive functions such as attentional allocation and the modulation of sensory processing[17]. It was from the mid-2000s when furthermore, evidence from intracranial and scalp EEG recordings during pharmacologically controlled interventions in rodents and secondarily, in some spare clinical models benefiting from intracranially implanted patients (SEEG and DBS in epileptic or Parkinson's patients), showed for the first time causal cell-type-specific activity linked distinct electrophysiological signatures[24]. Consequently, oscillatory rhythms do not solely inform about local neural activity but, can also be associated with predominant cell-type-specific activity and highlighted as key phenomena underlying system-specific (excitatory/inhibitory) interactions linked to specific human cognition. Moreover, communication and processing dysfunctions (oscillatory dysfunctions, also referred to oscillo-pathies) may also underlie some of the symptoms classically associated to neurological conditions, providing ground for network-directed interventions in neurological disorders.

1.1.2 Non-linear communication mechanisms

During the last decade, progress has been made in characterizing neural synchrony processes beyond quantitative estimations of oscillations in terms of power measurements (a simple measure of the relative amplitude of a given frequency band within a spectrum and time window of reference in decibels). Additional layers of complexity pinpointing the mechanisms by which neuronal populations communicate, demonstrate that single cell communication is highly influenced by local and regional resonant rhythms. Currently, a fair account **of neural communication models includes the *input-output* gain function**[24–29]**, the communication through coherence**[19]**, the cross-frequency coupling (phase-to-frequency, phase-to-phase, phase-to-amplitude or amplitude-to-amplitude)**[30–33]**, the gating by pulsed inhibition**[34,35]**, phase procession, entrainment or phase-dependent plasticity**[36–40]**.** The first and most classical of them all, the *input-output* gain function model states that spontaneous fluctuations of neocortical excitability unfold via the so-called multi-frequency hierarchically nested oscillations so that oscillatory states at the mesoscale orchestrate in a phase-dependent manner the timing of information flow. It

follows (and so was experimentally shown) that, stimulus conveyed to the brain via sensory visual or tactile afferent patterns or delivered to the human cortex, aligned with specific phases (either during or in the vicinity of to its peak or trough) of relevant ongoing cortical oscillation generates in higher levels of neuronal recruitment (pulsed facilitation) than those delivered at the antiphase[25].

Building on this principle, Varela[18], Buzsáki[17] and Fries[19], proposed the first models of *communication through coherence* (synchronization), suggesting that effective communication and synaptic strengthening between neurons would occur when neuronal population exchange information in a precise phase-synchronized manner. The so called *coherence model* was based on the following key insights: first, activated neuronal groups were provided with the wired-in property to fluctuate (oscillate) periodically; and second, that those fluctuations resulted in periodical modulations of neuronal/neural excitability that affected both the sensitivity to synaptic input and the likelihood of generating a spike. If these two conditions were fulfilled, then rhythmically fluctuating/oscillating excitability peaks generated by such systems, set up recurrent temporal windows for optimal communication or the lack thereof in a fluctuating manner. It derived that only coherently oscillating (phase-locked) neuronal populations may communicate effectively because their communication windows (polarization levels) for accepting inputs and generating outputs were open (or in 'ready' mode) at the same time. On that context, the precise timing of events (event frequency) becomes critical for effective communication across populations and the oscillatory frequency of a receiving (*input*) group needs to match the one of the sending (*output*) group. However, for a given set of anatomically connected regions conduction delays might impact *input* transmission hence different oscillators can have peaks of optimal communication coherence at different phases. Delays are generally an order of magnitude shorter than the oscillatory cycle length (e.g., for example, oscillations at the gamma range 30 to 100Hz have cycle lengths of 10 to 30 ms and conduction delays of ~1-3ms, whereas beta oscillations from 15 to 25hz have cycle lengths of 50ms and conduction delays of ~5ms). However, different neuronal cell-types can be more sensitive to specific frequency inputs and at the same time their sensitivity to inputs might be conditioned by their previous history of events[24].

Oscillators can also phase synchronize across amplitudes, frequencies and phases and indeed, cross-frequency communication has been established as a mechanism for local information processing across cortical and subcortical top-down and bottom-up networks. For example, the phase of fast oscillatory modes can drive the amplitude of slower ones and vice-versa: this is known as hierarchical *cross-frequency phase-amplitude coupling (PAC)*. In the context of such family of coupling phenomena, the phase of low-frequency oscillations can be synchronized not only with the *phase* but also with the *amplitude*, of another (high-frequency) oscillation[31]. Moreover, it has been suggested for example that the phases of low-frequency oscillations reflect synchronized fluctuations of resting transmembrane potentials from neurons hosted in a given brain area. These fluctuations represent changes in neural excitability, affecting the amplitude of high-frequency oscillations in the same area. Therefore, the phase of slower rhythms can encode (i.e., phase coding/procession) sensitive time points where faster rhythms are more likely to emerge. Phase-to-phase synchronization can also occur between oscillators operating at different frequencies. This addresses the phenomenon that not only entire cycles but even within the individual phases of single cycles with higher

frequencies are locked to specific phases of a slower-frequency oscillator. This mechanism is more likely to reflect neuronal communication scenarios in which information transfer needs temporally adjacent but non-overlapping representations of multiple stimuli. Indeed, variants of cross-frequency coupling have demonstrated biological relevance such phase-to-phase coupling (PPC defined as coupling between phases of two oscillations, also known as phase locking value or coherence), phase-to-frequency coupling (PFC, understood as the coupling between phase of one oscillator and the amplitude of a second one) or amplitude-to-amplitude coupling (AAC, characterizing the coupling between the amplitudes of two oscillators) (Figure 4). Even if these communication models have been theoretically characterized and has been observed between slow and fast oscillators, further experimental work is still required better understand their biological implications.

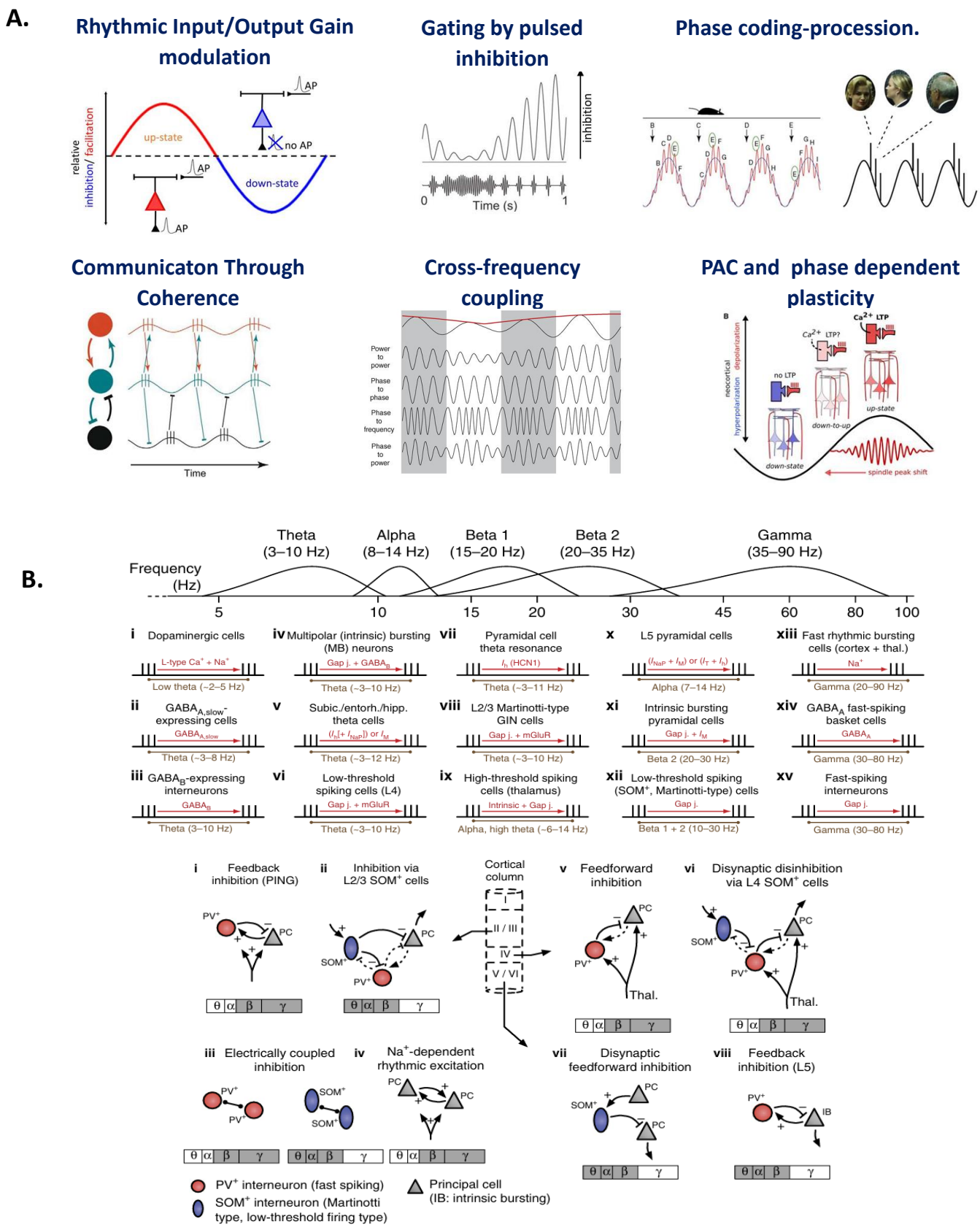


Figure 4. Illustration presenting current neuronal communication models and circuit mechanisms underlying rhythmic control, gating and information integration. A. Activity-driven mechanisms of neuronal communication driven by mesoscale oscillatory coupled rhythms, with implications for information processing and neural plasticity. **B.** Empirical evidence supporting models of cellular and functional mechanisms subtending rhythms at different frequencies. Adapted from *Bergmann TO* and other sources [19,24,30,34,307].

It has also been proposed that the transfer of information is routed by functionally blocking/downplaying irrelevant pathways and stimuli, a process known as *gating by inhibition*, subtended, for example, by increases of oscillatory activity in the alpha range (7-13Hz), which was one of the first oscillatory rhythms specifically associated with an inhibitory role. Indeed, oscillatory activity in the alpha band was first reported in the 1920s by the discoverer of scalp EEG, Hans Berger, and associated for a long period to the notion of 'cortical idling' particularly noticeable when we close our eyes. However, more recent investigations shown that alpha rhythms decrease in engaged regions, while increase in disengaged regions during behavioural tasks, suggesting that optimal task performance is dependent on 'silencing' task-irrelevant regions (by increasing alpha activity) in order to allocate resources to task-relevant regions[34]. To this same regard, it has been proposed that alpha activity is the consequence of an increase in the magnitude of inhibition oscillatory signals via rhythmic GABAergic input from an intracortical network specifically enabling pulsed inhibition[35]. Following this principle, by blocking ongoing higher frequency activity representing neuronal-to-neural communication) in a phasic manner, alpha activity could implement graded inhibition.

Deepening our understanding of this specific band, particularly relevant for this specific dissertation, into lower level molecular processes, alpha inhibitory activity is thought to partially rely on low-threshold T-type voltage-gated calcium channels[41] expressed in somatostatin positive GABAergic intracortical interneurons, which exhibit alpha-compatible slow dynamics. Accordingly, increase of local alpha activity could suppress processing in pyramidal neurons or reduce the efficacy of excitatory input by shunting inhibition through cross-frequency interactions. However, depending on the synaptic microcircuit architecture, distinct frequency patterns of neural activation can be recorded, and non-inhibitory mechanisms such as for example the activation of low threshold L4 pyramidal cells can result in similar rhythms to those classically associated with inhibitory activity[24]. Therefore, transcranial recordings of alpha activity may or may not play an inhibitory role depending on contextual frameworks.

Despite alpha activity being one of the most prominent and easily monitored oscillatory features in extracranial EEG recordings, a broad range of frequencies can be recorded and have been associated to cognitive or behavioural processes. For example, precise cerebello-thalamic-cortical synchronization at the beta band has been involved in movement generation and some associated movement disorders[42–45]; beta-gamma oscillations have been linked to visual processing[46], very low frequency oscillations and spindles have been shown to be critical during sleep for memory consolidation and wakefulness[47,48], whereas parieto-frontal theta-gamma coupling has been linked to working memory[49]. Moreover, distinct brain regions exert different natural oscillatory signatures according to the architecture of the circuit they are part of and the integrative cognitive framework to which they might contribute at a given time. Accordingly, nodes and networks might participate in multiple sequential or simultaneously co-occurring phenomena of local and distant neural communication modulated by local cytoarchitectural characteristics and control mechanisms. *Input-output gain function, local and long-distance coupling*, and *cross-frequency interaction* mechanism are among other processes key for an effective integration and routing of information in the healthy state, may prove dysfunctional in pathological situations explaining the emergence of neurological deficits; last but not least, may contribute to

the long-term shaping and re-organization of the structure and function of the nervous system via neural plasticity.

1.1.3 System reorganization: plasticity

The central nervous system is capable of adapting structurally and functionally in response to stimuli, whether external or internal, through changes that operate across organization scales: molecular, cellular, organs and distributed systems. Such modifications, either short or long-lasting triggered by activity changes were termed *plasticity* by Donald Hebb in his seminal 1949 postulates[6]. Along this same vein, **the brain is a dynamic organ in continuous change where every sensory input, motor action, association, reward signal, action plan, or actual behaviour has the potential to reshape neuronal communication, and vice versa**. Plasticity can be either adaptive and contribute to a better (re)adaptation to challenging changes of our environment, or maladaptive and leading to cognitive dysfunction in the context of aggravated neurological or psychiatric disorders.

Beyond those classical scenarios, plasticity can also be viewed as a 'therapeutic playground on which to operate to supress maladaptive processes while boosting others that provide a behavioural benefit and better adaptation to an internally or externally changing environmental demands. To this regard, Hebb predicted a form of synaptic communication driven by temporal contiguity, conditioning pre- and postsynaptic activity and anticipating 30 years the phenomena of short and long rage synchronization and its effects on synaptic strengthening. His famous assertion, 'cells that fire together wire together,' encapsulated this idea. More than two decades later, Timothy Bliss and Terje Lømo conducted the first experiments on anesthetized rabbits *in vivo* models exploring the short- and long- term effects of pre-and post-synaptic conditioning, describing for the first time the long-term potentiation (LTP) in entorhinal-hippocampal networks[7]. Working together at the laboratory of Per Andersen at the University of Oslo in 1973 they reported for the first-time that high-frequency presynaptic activity within a temporal window generate a progressive and sustained facilitation of postsynaptic responses (dubbed LTP), whereas the reversed pairing or a decrease of stimulation frequency would result induce the opposite effect (Figure 5). A decade later, in 1982 similar work performed in cerebellar slices by Masao Ito at the Riken institute in Japan showed that repetitive stimulation of parallel fibres and climbing fibres in the cerebellum led to a long-lasting decrease in the efficacy of parallel fibre synapses onto Purkinje cells a phenomenon that was named long-term depression, (LTD). Importantly, such discoveries laid the foundation for decades of neuroscience research where multiple types of electrical and neurochemical communication mechanisms at the neuronal level leading to sustained plastic changes were characterized, nourishing the notion that activity-driven modification in pre-existing synaptic connections depends on the precise timing and patters of activity in the pre and post-synaptic neurons. While progress had been made since Hebb’s postulates, the mechanisms, impact, and malleability of these phenomena in human cortical networks, especially in relation to enduring or reversible plasticity, remain incompletely understood.

Major forms of short-term plasticity, lasting from milliseconds to minutes, are known to play important roles in short-term adaptations to sensory inputs, transient behavioural states and short-lasting memory. Most forms of known short-term plasticity are triggered by short bursts of activity causing a transient accumulation of calcium in presynaptic terminals[50–53]. This increase in calcium, which may result from changes in voltage-gated channels at the cellular membrane level, alter the probability of neurotransmitter release by directly modifying the biochemical processes underlying the exocytosis of synaptic vesicles. Major forms of activity-dependent short-term plasticity can be made evident by paired-pulse facilitation/depression local phenomena following pairs (combining a conditioning pulse priming the output of a test pulse at short or long interstimulus intervals) or trains of stimuli, or via the modulation of transmission efficacy by presynaptic receptors. In rodent models, paired-pulse depression, occurring at short interstimulus intervals (less than 20ms), likely results from the inactivation of voltage-dependent sodium or calcium channels or a temporary depletion of vesicles ready for release at the presynaptic terminal. Conversely, paired-pulse facilitation is seen at longer intervals (20–500ms) and may be due to residual calcium from the first action potential contributing to the second stimulation, alongside other potential mechanisms involving protein kinases and presynaptic phosphoproteins[52]. Interestingly, similar phenomena of intracortical facilitation or inhibition have been reported at short (1-4ms) and long intervals (10-15ms) in the human primary motor cortex as tested with transcranial magnetic stimulation (TMS) and motor evoked responses (MEPs).

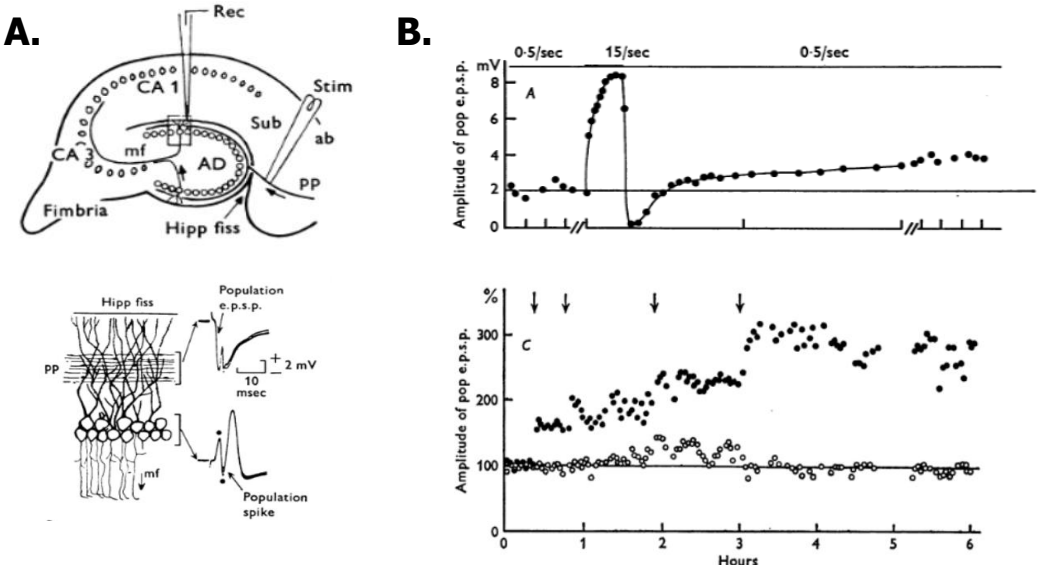


Figure 5. Illustration of original LTD experiment by Bliss & Lømo. Original experimental reports from Bliss & Lømo. **A.** Diagram of the parasagittal section of the hippocampal recorded and stimulated circuits. **B.** Superior panel illustrate results from a potentiation conditioning train (15Hz for 15 seconds) in the amplitude of the population excitatory post-synaptic potential (EPSP). Lower panel denotes long-lasting and summatory effects over time of conditioning trains (15Hz for 10 seconds) delivered at different time points (black arrows) (Adapted from[7]).

Similarly, short-term plasticity can also occur following repetitive or tetanizing stimulation (i.e., high frequency electrical pulses allowing minimal to none post-depolarization recovery time) of synapses following extended trains of stimulation. These phenomena can be made evident as augmentation or the so-called post-tetanic potentiation of muscle responses via the enhancement of neurotransmitter release at the level of the muscle junction by peripheral spinal motor neuron terminals lasting from seconds to several minutes (Figure 6). They involve an increase in the probability of transmitter release in response to a train of action potentials caused by a buildup of calcium concentration in the presynaptic terminal during the stimulus trains. This residual calcium may combine with the calcium influx elicited by the subsequent single action potential to directly enhance the release of neurotransmitters or lead to biochemical modifications of proteins in the presynaptic terminal. In some synapses, repetitive activation leads to depression, thought to result from a transient depletion of a 'release-ready' pool of synaptic vesicles, desensitization of ligand-gated receptors, making the target neuron less sensitive to neurotransmitters. Then, the release of modulatory compounds from the activated presynaptic terminals, postsynaptic, or neighbouring cells, initiate a signalling cascade that leads to inhibition of the presynaptic release machinery. Many of the above-mentioned short-term plasticity mechanisms are nowadays used as experimental models in animals and humans to explore neural communication and the integration of synaptic information under experimental scenarios where genuine LTP/LTD phenomena or 'LTP/LTD-like' are induced.

On the other hand, long-term synaptic plasticity, lasting in the order of minutes to days, are thought to play a key role in long-term memory formation and human evolution. To this regard, several subtypes of long-term plasticity have been characterized over the last decades from the originally *LTD and LTD* phenomena (also referred to as *Hebbian plasticity*), to other forms of plasticity such as *synaptic scaling*, *spike-timing dependent plasticity (STDP)* or *synaptic redistribution* (also called non-Hebbian). Hebbian LTP and LTD are considered to be a 'positive feedback-driven' process because effective synapses are strengthened or weakened as a function of pre-synaptic inputs. In contrast, non-Hebbian forms of plasticity, such as *synaptic scaling*, does not operate under those same assumptions and modifies synaptic strength in a inversely proportional manner regarding the generated post-synaptic activity, adjusting synaptic strengths based on the overall postsynaptic firing rate. Additionally, the precise timing at which pre-and post-synaptic depolarization occur can drive total net LTD or LTP effects. For example, in the so-called Hebbian *STDP*, LTP occurs when presynaptic spikes precede postsynaptic spikes by 0 to 20ms, whereas LTD is induced when postsynaptic spikes lead presynaptic spikes by 0 to 20–100ms. In the so-called anti-Hebbian *STDP*, LTD occurs when presynaptic spikes precede postsynaptic spikes. In a few cases, post-leading-pre spiking also drives LTP, resulting in bidirectional STDP opposite to Hebbian STDP. Importantly, STDP depends not only on spike timing, but also on firing rate, synaptic cooperativity (need for multiple coactive synaptic inputs to generate sufficient depolarization), and postsynaptic voltage. Finally, STDP also depends on baseline synaptic weight. These requirements demonstrate that a single postsynaptic somatic spike is not a sufficient signal for associative plasticity, and that multiple spikes are required, and these must interact with local dendritic depolarization phenomena produced in part by spatial summation of local synaptic potentials, in turn highly influenced by ongoing resonant rhythms.

1.1.4 Input-output models of human plasticity

Plasticity in the human brain has been evaluated through several neuroimaging tools that assess either structural changes (such as whole-brain or regional volume) or functional connectivity (strength of coupling) often in individuals with a high-degree of training and expertise in a specific activity (musicians, sports-men/sport-women, taxi drivers, air controllers etc.). Unfortunately, the limited temporal resolution (in the order of 2-5 seconds) and spatial resolution of standardized 3T MRI based neuroimaging modalities (with voxel sizes between of ~1 mm^3) makes them unable to directly extract information about changes in neural connectivity/communication of local or extended systems driven by plasticity mechanisms. As a result, most of the aforementioned models of plasticity have been explored experimentally in the human brain employing noninvasive supra-threshold stimulation approaches, at intensities enabling the generation of action potentials combined with EMG, perceptual reports and most recently EEG recordings. In such endeavor, the primary motor (M1) and the primary visual (V1) systems have extensively served as 'test bench' given the facility to induce and characterize non-invasively with TMS single pulses physiological responses, used as biomarkers of local excitability. As a result, most of our current knowledge on short-term responses is limited to local networks in such areas, precluding our ability to generalize our findings to low-order or higher-order regions.

Similar to *in vivo* experimental protocols in rodents that evaluate post-synaptic potentials following stimuli or stimulus trains, supra-threshold transcranial neurostimulation, and notably TMS, has enabled for the last 30 years to explore the modulation of local plasticity phenomena via pattern-dependent stimuli. **Currently, stimulation modalities with an ability to modulate neurophysiological properties associated to plasticity phenomena can be designed by operating on: (1) pulse number and multipulse burst duration, (2) time interval between pulses or trains of pulses organized in bursts and (3) inter-pulse or inter-burst discharge frequency.**

For example, the amplitude of peripheral muscle responses, known as motor evoked potentials (MEPs), evoked by single pulse TMS protocols has been used to directly index cortical excitability levels of M1 and their associated descending projections. Cortico-cortical paired-pulse protocols, with a conditioning pulse delivered outside the M1 area probed by the test stimulus in a M1 of interest (e.g. the premotor cortex PMC, the intraparietal sulcus IPS, the supplementary motor cortex SMA, the inferior frontal gyrus IFG eventually the contralateral M1) at different temporal delays have allowed for the verification of a connection, estimate their cortico-cortical conduction time and gauge their functional strength and excitability[54]. Finally, *Input-output* curves understood as M1 dose-response functions relating TMS pulse intensity and MEP amplitudes, corticospinal silent period measurements estimating the duration of EMG suppression following M1 single pulse stimulation, and more recently, cortical mapping with EEG of transcranial evoked potentials (TEPs), have all been used to study cortical excitability and signal transmission[55], and provided in direct or indirect manners diagnostic biomarkers for neurological conditions.

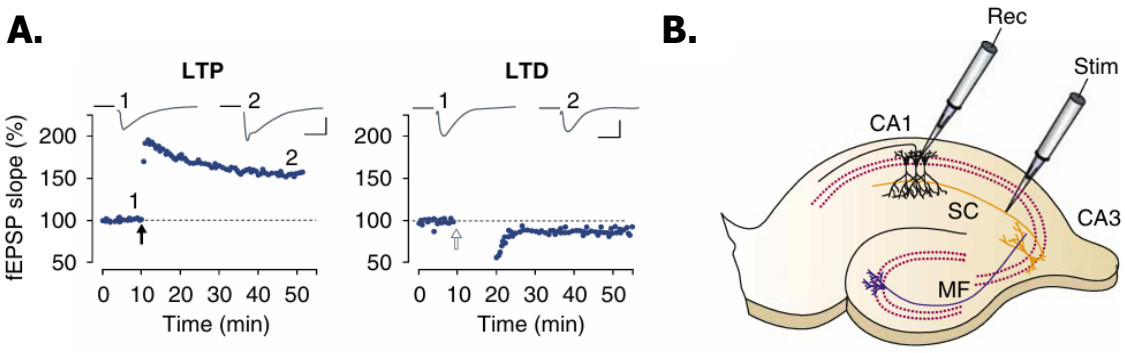


Figure 6. Prototypical LTD and LTD modern reproduction. **A.** Synaptic strength (evaluated via the amplitude of excitatory post-synaptic potentials) plotted as a function of time. The left panel shows an example of LTP elicited by high-frequency tetanic stimulation (100 Hz for 1 second). The right panel illustrates LTD phenomena elicited by low-frequency stimulation (5 Hz stimulation for 3 minutes). **B**. Schematic diagram of the rodent hippocampal slice preparation used for conditioning plasticity experiments (SC: Schaffer collateral; MF: Mossy fiber) (Adapted from[51]).

Nonetheless, one of the most sensitive indices of GABAergic and Glutamatergic influences by intracortical interneurons has been frequently explored through double-pulse TMS paradigms. Also known as paired-pulse, this technique discharges to the same exact M1 region a suprathreshold (sometimes subthreshold) test stimulus (TS) able to induce sufficient and consistent MEP output, preceded by a subthreshold (sometimes suprathreshold) conditioning stimulus (CS) separated by an interstimulus interval (ISI) which can be long or short[55]. At short ISIs (<7ms, optimally between 1-4ms) intracortical interneurons conditioned by the CS require time to recover baseline depolarization capability after a refractory period will not be immediately fully responsive, meaning the TS will activate only a limited proportion of neurons resulting in an MEP of lower amplitude compared to the one generated by the TS alone. In contrast for long ISI (= or >10ms up to a maximum of 15-18ms), the same CS facilitates intracortical mechanisms by aligning the TS to post-potential depolarization rebounds, which increase the number of recruited units and the size of MEP outputs compared to those induced by the TS alone[56–61]. Extensive research using a large variety of short (<7ms), intermediate (7-15ms) and long (50-200ms) ISI intervals has revealed in M1 up to 5 different types of intracortical modulation phenomena: *Intracortical facilitation, ICF* (10-15ms ISI, with a subthreshold CS and suprathreshold TS), *Short interval intracortical inhibition, SICI* (1-4ms ISI, with a subthreshold CS and suprathreshold TS) the two most conventionally employed and in addition, *Short interval intracortical facilitation, SICF* (1-4ms ISI, with a suprathreshold CS and a subthreshold TS), *Long interval intracortical facilitation, LICF* (100ms ISI, subthreshold CS and suprathreshold TS), and, *Long interval intracortical inhibition* LICI (40-200ms ISI, with a suprathreshold CS and a subthreshold TS). Studies combining paired-pulse TMS and systemic pharmacological interventions at low-doses suggest that effects of short ISI on TS-induced MEP inhibition would be mainly mediated by a release of gamma-aminobutyric acid (GABA) and/or a 'shunting inhibition' caused by an increase in transmembrane conductance which accompanies the classic GABAA inhibitory postsynaptic potential (IPSP). In contrast, long ISI intervals resulting in TS-induced MEP facilitations would be mostly mediated by glutamatergic intracortical mechanisms. Indeed, differences between SICI and SICF

have evidenced that across a continuum of ISIs, a CS may activate preferentially different circuits of interneurons (excitatory vs. inhibitory, as a function of their threshold, conduction time and length), hence modulating differentially cortical activity induced by the TS[60,62–68]. Other paired-pulse combining intrinsic TS on M1 with an extrinsic (exogenous) stimuli at increasing intensity levels following similar principles as the *input-output* measurements encompasses Short Latency Afferent Inhibition (SAI), Long Latency Afferent Inhibition (LAI) and Cerebellar-to-cortical Inhibition, CBI[55,69–73].

Finally long patterns (from 10 to 28 minutes for conventional repetitive TMS and 40 to 190 seconds for patterned TMS, aka. Theta Burst Stimulation) made of bursts of TMS pulses at conventional high intensity (80-90% of the resting motor threshold for conventional rTMS or 80% of the active motor threshold for TBS) have been extensively used to modulate human cortical plasticity and induce LTD or LTP-like effects (Figure 7). Repetitive TMS trains (the so-called repetitive TMS stimulation, rTMS) of pulses over a single cortical region can induce long-term effects, and either facilitate or suppress activity for a period outlasting the delivery of the pulses themselves[12,74–79]. Depending on TMS burst frequency and TMS inter-burst intervals, the suppression or facilitation will be more likely to occur. For example, experimental research in humans has shown that conventional repetitive TMS using a continuous low-frequency pattern (e.g.,1Hz) applied for 20-28 minutes, delivering a total of 1200-2800 pulses, suppresses the amplitude of motor evoked responses (LTD-like effects).

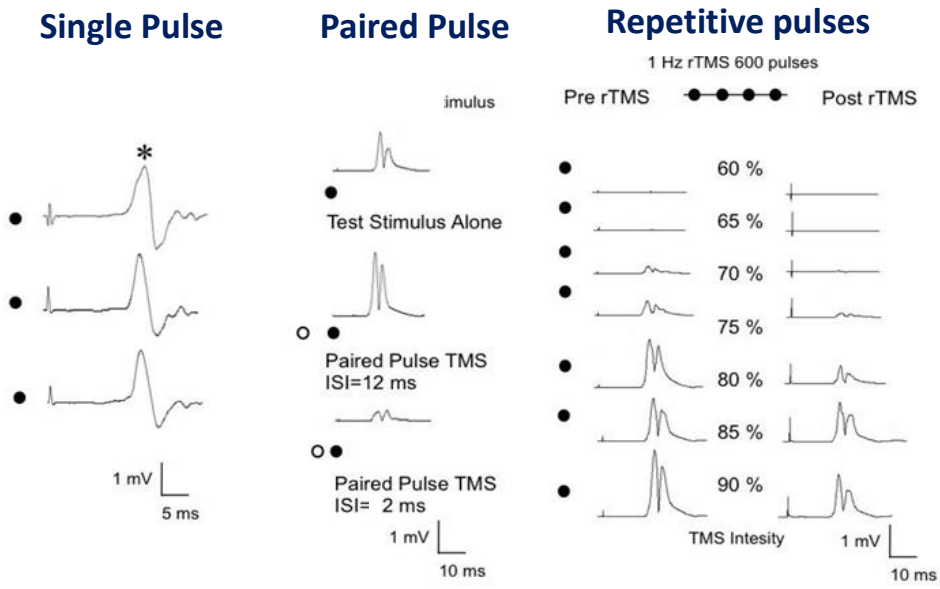


Figure 7. Illustration of short-term plasticity in humans. Example of subtypes of potentiation or suppression of corticospinal outputs such as Motor Evoked Potentials (MEP) from human recordings. In this example, electromyographic (EMG) activity is recorded from the right first dorsal interosseous muscle in response to stimuli delivered with transcranial magnetic stimulation (TMS) to the left primary motor cortex (M1). Single-pulse stimuli evoke consistent responses. The paired-pulse paradigm, employing pairs of subthreshold conditioning stimuli (CS) and suprathreshold test stimuli (TS) with a 12ms inter-pulse interval, induces facilitation. Conversely, a 2ms interval results in suppression. When repetitive trains of stimuli are administered at 1Hz for at least 10 consecutive minutes, suppression of evoked amplitudes is observed (Adapted from[54]).

In contrast, high frequency (5-25Hz) stimulation applied in short trains of 1-4 seconds with intertrain intervals of 20-40 seconds leads to MEP amplitude increases

(LTP-like effects). The impact of the inter-train interval is most evident in Theta Burst Protocol family, which delivers triplets of pulses (3 pulses) at 50 Hz (hence every 20 ms) repeated every 200 ms (hence at a 5 Hz frequency). For example, continuous TBS or cTBS (3 TMS pulses at a 50 Hz gamma frequency, with an inter-burst interval of 200 ms, hence repeated at theta 5 Hz frequency) delivered continuously for a period of 40 seconds will suppress corticospinal activity. In contrast, the same bursts (3 TMS pulses at 50 Hz gamma frequency) at the same inter-burst frequency (hence every 200 ms repeated at 5 Hz theta frequency,) but with an 8-second inter-train interval following 2 second blocks of active TMS stimulation would result in corticospinal facilitation. All these different patterns of activity will drive biological and functional changes imposing or enhancing frequency-specific local resonant rhythms able to drive activity dependent cellular and molecular cascades relevant for plasticity.

Nevertheless, taking into account combinations of the most common TMS parameters used to design conventional repetitive stimulation patterns (e.g., pattern frequency, inter-pulse interval, inter-burst interval, burst length, number of pulses per burst or per regime, and eventually in some TMS equipment also pulse shape hence monopolar or bipolar and pulse width), and the multiple tools employed in experimental neuroscience to silence or activate connections (magnetic fields, electric currents, genetically encoded sensors, focused ultrasound, etc.), an infinite number of potential configurations arise. Moreover, given differences in the cytoarchitectural, neurochemical and neurophysiological organization across cortical areas, and of the ongoing activity (TMS pulses delivered to a brain ‘at rest’ or in a ‘task-activated’ brain) at the time of stimulation, it is unlikely that the effects of a combination of TMS parameters may remain constant across conditions, participants or brain regions. Experimentally exploring such a humongous parameter space with an exploding exponential number of potential parameter combinations and crossed an equally vast space of variables influencing fluctuating brain states, differences in experimental setups and protocols for every specific brain region, seems an impossible mission. In such context, computational neuroscience approaches combining biophysical current distribution predictions with neural mass models integrating cytoarchitectural, neurochemical and structural and functional anatomical features and input-output response predictions of under specific conditions of physiological or pathological ongoing activity has become of paramount[80–86].

1.1.5 Stroke and brain damage: a disconnectome perspective

Stroke is the most common cause of disability in adult humans leading to a wide variety of either transient or enduring deficits depending on the impacted brain systems and affected function. The resulting neurological deficits tend to have an impact on the patient’s daily activities, their ability to reintegrate into the workplace and his/her quality of life, to which one needs to add the financial burden posed by health costs and the required adaptations[87,88]. Difficulties and challenges encompass life-threatening human functions such as an impact on respiration, heart rhythm, blood pressure and the control of peripheral vessels, and high-order cognitive processes such as motor function, attention, language, memory, impulsivity, mood, motivation and goal-directed behaviours. Despite the notorious influence of lesion site, size, volume on the type,

magnitude and prognosis of post-lesional symptoms, given the natural functional arrangement of the human brain organized across large scale functional correlated and anticorrelated networks (Figure 8), brain deficits following stroke cannot be solely explained by the loss of function in areas with direct ischemic or haemorrhagic vascular damage but also connectivity-mediated functional *diaschetic* effects caused by dysfunctional interactions with local and distant network structures contributing adaptive activity, sound communication and adequate information processing[89–92]. Within this *diaschetic* damage framework, similar lesions of the same brain region can develop distinct functional deficits in different brains depending on the ability of spared connectivity patterns and areas to mediate adaptive plasticity. Likewise, lesions in different brain regions may converge in the same set of neurological deficits, since[15] they share common networks via white matter bundles connecting to central nodes critical to sustain sound function.

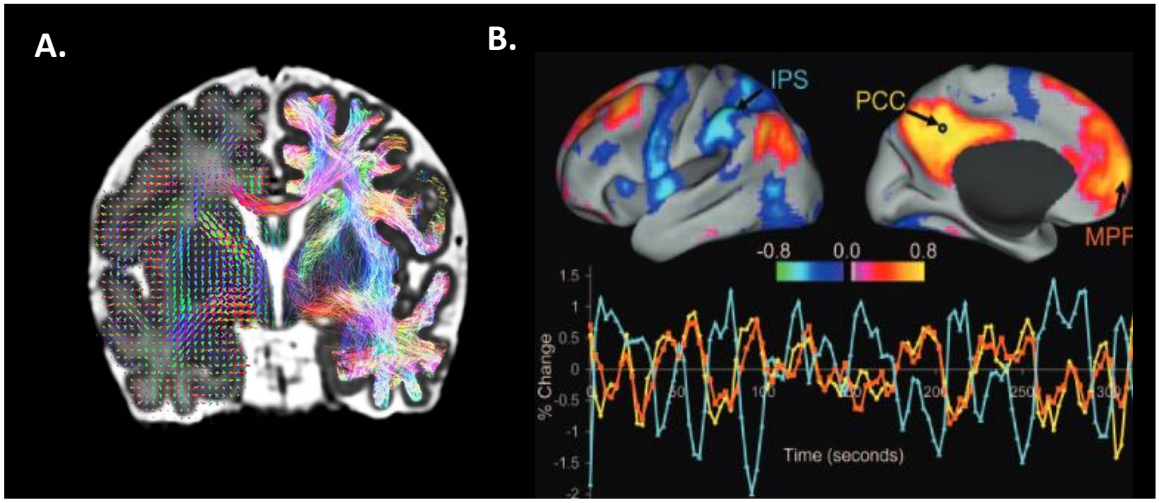


Figure 8. Graphical illustration of the notion of structural and functional brain segregation. Left Panel (A). Coronal brain section at the level of the frontal cortex showing diffusion imaging structural connectome images with predicted anatomical white matter bundles derived from diffusion tensor imaging (DTI). **Right Panel B.** Representation of intrinsically anticorrelated resting state fMRI networks, extracted from functional magnetic resonance imaging (fMRI) (Adapted from[288,308]).

Following brain damage, recovery by compensation or remapping of lost functions requires the spontaneous or induced reorganization of the central nervous systems. Research effort have been invested in the study of methods to manipulate adaptive plasticity, to accompany and facilitate functional reorganization and to promote re-adaptation. However, beyond early-stage thrombolysis and thrombectomy, decompression via craniectomy for large haemorrhagic strokes paramount to limit the extent of the damage across the penumbra area surrounding the ischemic core, and conventional neurorehabilitation activities with inconsistent and variable impact, effective interventions to optimize recovery have been lacking for a large majority of stroke patients. In this context, novel interventions aiming to guide and boost spontaneous plasticity have been gathering momentum. One of the major limitations to pave the path to success for such an ambitious purpose, is to further our comprehension of the role that different cell types, including neuronal and non-neuronal glial units and tissular, ionic and neurochemical component spilled over in the extracellular space, compiling a detailed pathophysiological model. Stroke effects are not limited to neurons but involve other brain cells and extracellular matrix components conforming a neurovascular glial

niche/scar that engages a cascade of molecular signals calling for a key inflammatory reaction mediated by the immune system[93–103]. Indeed, quite early following a stroke event (from minutes to hours), endogenous neuroprotective mechanisms are activated. Brain infarcts generates an imbalance in energy demand and blood supply leading to the interruption of synaptic transmission accounting for the electrical silence observed in the penumbra region after the stroke. Hypoxic failure results in focal postsynaptic hyperpolarization followed by a massive hypoxic depolarization of the postsynaptic terminals. Synaptic disinhibition failure anticipates synaptic excitation due to reduced excitatory input to inhibitory interneurons, potentially contributing to a phase of Glutamate excitotoxicity that causes delayed cell death.

Consequently, activation of anti-excitotoxic channels and regulators like γ-aminobutyric acid (GABA) or K+ charge channels and coupled transporters followed by a transduction phase with the amplification of kinases, transcription factors and growth factors (e.g., Neurotrophin 3, Granulocyte Colony-Stimulating Factor and Vascular Endothelial Growth Factor) together with brain-derived neurotrophic factor (BDNF). Similarly, increased molecular concentrations of anti-apoptotic proteins, anti-inflammatory and anti-oxidative factors are released intracellularly to enhance cell survival and lately promote neural repair. If those mechanisms are effective in acute stages and proper clinical care is provided, chances of cellular survival are high and previously described normalization of cellular baseline activity may occur[104–107]. Nevertheless, functional compensation is automatically triggered on the surviving tissue participating to the reorganization of initially damaged nodes and associated networks. In rodent stroke models, it has also been reported a increase of neurogenesis from neural stem cells which differentiate in neuroblasts migrating to the lesion *core*[104], however it is unclear how much such effects may play out in sophisticated mammals as humans. Neuroblasts will differentiate into mature neurons and enhance angiogenesis. Additionally, glial cells and astrocytes have been involved in removing an excess of excitatory neurotransmitters (notably glutamate) limiting excitotoxic damage. Facilitatory circuit remodelling effects occur in sub-acute phases, pointing at the post-acute time-window post-stroke (days to 3-6 months post stroke) as the optimal period for inducing external rehabilitation or brain stimulation interventions (Figure 9).

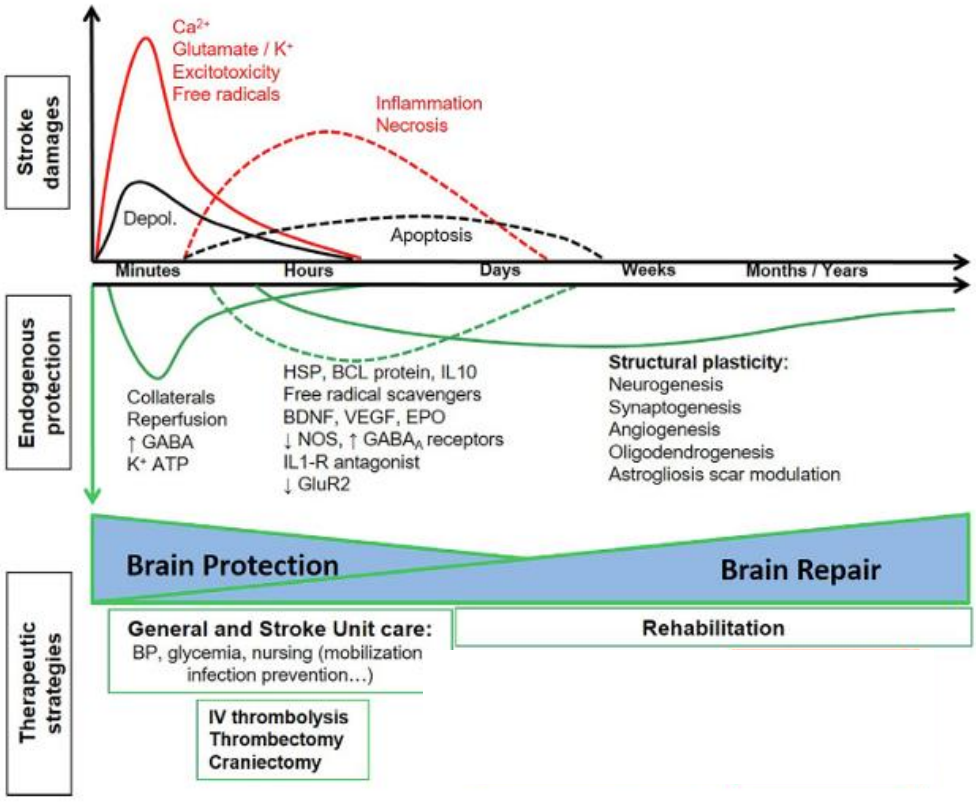


Figure 9. Diagram of cellular cascades triggered by stroke events. Schematic illustration of dynamic mechanisms triggered by stroke brain damage and endogenous protection facilitating functional repair (Adapted from[93]).

Importantly, despite genetically encoded organization prevails with pre-established assignments, functional and structural organization of brain networks can be flexibly remodelled by plasticity. As mentioned, this wired-in property is a general term often used to describe how a neuron, a neuronal network within a brain region or a network of regions/structures expands the restrictions set by our own genome and change their structure or the functional efficacy of their connectivity pattern. **From a physiological perspective, plasticity requires the reorganization of neural connections and subsequent or the reorganization of cognitive functions, via the strengthening or weakening of pre-existing or novel synapses based on the influence their firing history** [108]**.** Nevertheless, despite engaged by a different and unique combination of stimuli throughout the lifespan of a human being, plasticity is present and naturally modulated at all stages of brain development and aging[109].

The term ‘reorganization’ describes a compensatory functional shift in neuronal populations or brain regions which are not necessarily spatially allocated contiguously to the region of interest, connecting through medium or long-range projections or even indirectly connected situated in other functional nodes. The term ‘remapping’ refers to a process by which cerebral representations (neuronal maps) following a change of input/output activity patterns or a central/peripheral lesion, implement functional attributions in contiguous neuronal populations different than those originally assigned them, in an attempt to overcome the maladaptive consequences of such effects. Both

remapping and reorganization can be understood as compensatory mechanisms to face changes in input-output processing and resulting in novel previously non-existing functional outputs different. It is well-known that such changes are particularly intense during the so called special 'critical periods' such as development. Despite the general notion of brain plasticity as the generation of new connections or the integration of new neurons *via* neurogenesis (still controversial in aged humans), plasticity in the adult human brain may be principally articulated by the unmasking of latent connections and neurons, tuned cells and re-direction of computational capacities[108].

Paradoxically, plasticity theories and subsequent clinical rehabilitation or interventional protocols in the field of sensorimotor recovery have been nestled in highly influential studies carried out during the 80s and 90s in the somatosensory, auditory, visual and motor systems by pioneering neuroscientists such as Jon Kaas, Michael Merzenich, Jerome Sanes and John P. Donoghue, to name a few. Particularly important in the sensorimotor systems was the seminal work of Randolph Nudo and colleagues (Fig. 9, Experiment 1)[110] who characterized for the first time plasticity processes underlying motor recovery following a stroke and rehabilitation in the non-human primate (Figure 10). On such studies, adult squirrel monkeys were pre-trained on a hand dexterity task and then small sub-cortical ischemic lesions (infarcts) were made within the M1 hand representation. Intense dexterity rehabilitative training was resumed 5 days after the cortical infarct and continued 3 to 4 weeks post-stroke until animals re-gained fine motor abilities. Simultaneously, intracortical microstimulation was used to derive detailed maps of changes of the hand muscle representation before the infarct and following 3 to 4 weeks of post-stroke active rehabilitation. Indeed, when compared with the pre-infarct map, cortical maps were modified and regions controlling the elbow and shoulder proximal movements were 'taken over' by digit and wrist evoked movements. These same authors, in another study (Fig. 9 Experiment 2)[111] performed the same type ischemic M1 lesions, however this time no rehabilitation was carried out and recovery to baseline performance was spontaneous taking 5 months. Importantly, cortical mapping revealed that there was a widespread reduction of the digit cortical representations with their apparent replacement by proximal muscle representations.

Thus, rehabilitation after brain damage may lead to cortical expansion[110–113], but this phenomenon is not necessarily critical and unique for recovery. Cortical expansion after stroke may be driven by rehabilitative training and may be an adaptive phenomenon, but plastic changes facilitating recovery may have multiple pathways and distinct mechanisms to emerge. Rehabilitation endorsing qualitative-guided interventions may be a contextual conditioning to drive adaptive plasticity. Without external conditioning, plasticity may occur, but maladaptive phenomena can dominate system-level changes and recovery of damaged function may take months. A quantitative change in the extension and level of granularity of cortical maps (i.e., structural plasticity) does not necessarily indicate a qualitative shift (i.e., functional plasticity) in the identity of a M1 neuronal population. Such studies suggest that recovery could be related to expansion of local cortical representations, but most importantly, functional recovery encompassed synaptic reorganization occurring in ventral and premotor regions and other nodes of the sensorimotor system, proving that the upregulation or strengthening of residual descending pathways from the cortex to brainstem and spinal cord aid the restoration of the behavioural deficit relying on the circuit (i.e., motor function). Thus, plasticity requires a qualitative shift on information

processing by which adapted stimuli can guide such process into injured structures, strengthening residual existing pathways in case normalization remains not plausible.

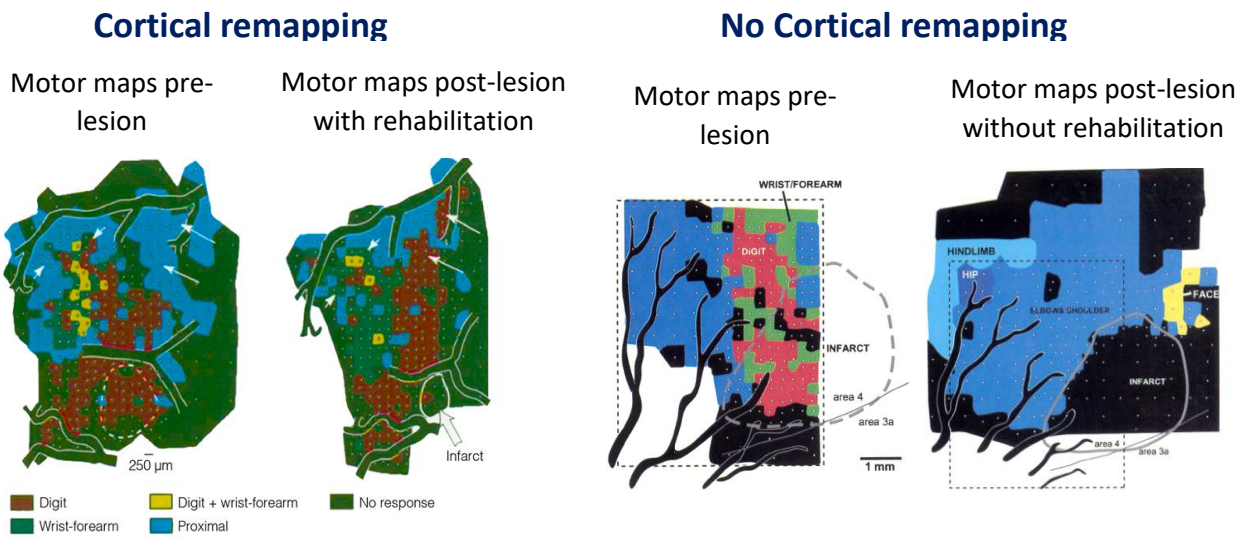


Figure 10. Illustration of cortical remapping in plasticity. Hand and arm representation following focal stroke of M1 in non-human primates. Left panel (experiment 1) shows cortical homuncular cortical maps elicited by M1 microstimulation (pre- and post-lesion) demonstrating signs of cortical remapping. Right panels show microstimulation maps (pre and post lesion) cortical maps (pre-post lesion) without rehabilitation, demonstrating no cortical remapping. However, subjects in both experiments recovered baseline levels of behavioural (motor) function challenging remapping as the mechanism underlying recovery (Adapted from[110,111]).

In general terms, early molecular, cellular and neurochemical cascades of events protect the CNS from further damage by locally promoting functional inhibition to neuronal cells. Over the weeks, inhibition is reversed by other processes promoting instead excitatory cascades and the promotion of synaptogenesis. Multimodal longitudinal imaging, via fMRI, TMS-EEG, magnetic resonance spectroscopy (MRS), electroencephalography (EEG), magnetoencephalography (MEG) and diffusion weighted imaging (tractography) have revealed that **early acute increases of the perilesional intracortical inhibitory tone is beneficial for prognosis in post-stroke sensorimotor human brain damage. However, depending on the system's resilience and its ability to reorganize function, perilesional inhibition can be beneficial or limiting for mid to long term (weeks to months post-stroke) prognosis** [42,89,114–125]. For example, in patients with mild to moderate lesions and functional motor impairments, early local up-regulation of GABAA-receptor function (inducing increased local inhibition) followed by a late downregulation function of the former and up-regulation of NMDA-receptor function (inducing late excitation) facilitates neuronal survival and adaptive plasticity. However, in patients with severe and extensive lesions and levels of motor paralysis with compromised recovery hence in need of plastic reorganization via distant structures, prolonged long-lasting local down regulation of GABAA-receptor function and distant up-regulation can be beneficial.

Unfortunately, maladaptive situations with suboptimal local/distant excitatory/ inhibitory imbalances for lesion recovery may often occur in clinical situations within regions of the primary affected networks. This is the case for example, in motor stroke

lesions impacting the contralesional hemisphere, by which inter-hemispheric imbalances occur, hence depending on lesion features and time after stroke, the contralesional hemisphere may play a supportive role for recovery or may contribute to maladaptive plasticity[126]. Teasing out adaptive vs. maladaptive contributions of specific patterns of activity and their effect on recovery remains a challenge. The so-called, *stratification models* based on *plasticity input-output interactions* have been proposed to better understand the role of the contra lesional hemisphere, the cerebellum and other secondary motor regions (e.g., premotor cortex, supplementary motor cortex, posterior-parietal cortex, striatum) quantifying the supporting adaptive or a maladaptive role on recovery[72,97,127–129]. These approaches are based on the notion that, the manipulation regional cortical excitability via LTD- or LTP-like effects with rTMS protocols (such as 1Hz or 10Hz respectively), can establish a causal contribution of a region in a tested function. Accordingly, to better pinpoint if a region is supporting or limiting recovery in a particular subject and time point of the post-lesional or recovery process, short-term transiently acting- excitatory/inhibitory protocols and ensuing changes in behavioural performance could serve to assess hence stratify the individual's ability for adaptative compensation and drive better individual therapeutic responses with NIBS.

Nonetheless, brain disconnections motivated by direct damage of the white matter tracts, or anterograde Wallerian degeneration of axonal fibres with compromised neuronal bodies (diaschisis) in grey matter areas, not only affect regions of the same functional network. Indeed, disconnections may simultaneously impact multiple cognitive domains (e.g., language, memory or motor planning). Accordingly, stroke patients generally present more than one deficit, whereas certain combinations of deficits occur more frequently than others, often structured in syndromes[114,116,130]. For instance, motor deficits, presumably largely dependent on a top-down corticospinal (and rubrospinal) tract, is known for being a strongly associated to structural damage. In contrast, speech and memory deficits, which rely on highly distributed patterns of cortico-cortical activity, may have a weaker relationship. For example, a lesion in the dorsal white matter portion of the right hemisphere underneath the motor cortex may cause hemiparesis due to structural damage of the descending corticospinal tract, but by local oedema, affect fibres of the dorsal attentional fronto-parietal network and induce attentional, orienting dysfunctions such as hemispatial neglect. As the oedema resolves, we would expect a faster and more significant recovery of hemineglect, decorrelating motor and attention deficits. However, If these two deficits are the behavioural output of a common desynchronization state, then similar time dynamics recovery will occur thanks to reorganization of neural communication between correlated or anticorrelated networks. Interestingly, focal lesions induce a low dimensional simplification of neural synchronization patterns, decreasing entropy and a decorrelation of inter-hemispheric dynamics and intra-hemispheric modularity. The normalization of these patterns correlates with recovery of the behavioural motor deficits[95,97,101,119,131–135].

Within the sensorimotor system, lesions on multiple brain locations can lead to motor disorders such bradykinesia, tremor, parkinsonism, hypotonic states, clonus, spasticity or often, given restricted M1 and corticospinal tract lesions, hemiplegia and motor paralysis. Stroke lesions on specific white matter tracts, like the corticospinal tract, are associated with particularly severe clinical deficits and lower levels of recovery, which has been shown with extensive corticospinal tract damage and motor function. Nonetheless, recent research employing fibre filtering and machine learning (ML)

approaches combining tractography (DWI) and causal data on cognitive impact generated with deep brain stimulation (DBS) has been able to identify the role of specific associative tracts and predict the effects of its deficits[16,136–139]. Unfortunately, white matter tracts (bundles of axons) within areas of stroke damage tend to retrogradely degenerate during the first weeks after stroke, leading to the death of neuronal bodies, hence an atrophy of spared and perilesional grey matter across regions and hemispheres, compromising clinical recovery and long-term prognosis. On the other side, stroke may also benefit from signal re-routing via spared structural connectivity within distributed networks, so that strengthened connections may compensate for the loss of original pathways. Adaptive plastic processes and/or adaptive compensatory plasticity mechanisms may benefit from critical substrates of damage-spared grey matter and white matter to operate and may result in spontaneous signs of recovery. Nonetheless, when let evolve alone, and depending on the type, localisation and volume of spared tissue, they may drastically fail or come to incomplete outcomes. In this context, an intelligent and anatomically and physiologically informed use of NIBS neuromodulation approaches, via TMS or tCS, can be considered to guide and boost natural processes of plasticity and increase chances for recovery.

1.2. Non-invasive brain stimulation

Effective translational neuroscience is grounded in two essential pillars which feed each other and are tightly interdependent each other: realistic concepts/models of brain function and efficient methods. Indeed, the development of solid mechanistic concepts and models grounded on detail anatomical and physiological facts is not feasible without a good understanding of methods and techniques used to achieve and solidify such knowledge. Conversely, the solid mechanistic concepts is essential to understand the potential and limitations of the information provided by such methodologies and optimize them to keep them enriching an accurately study of brain function and to develop realistic therapeutic applications for neurological disorders. In such context, the careful application of a method is an important step in the scientific journey. New methodologies have always led the way in developing new scientific disciplines in the biological and medical sciences, and non-invasive brain stimulation methodologies is not exception to this rule.

Thanks to their ability to either suppress, quantify, boost, perturb, interfere or modulate regional correlates of brain activity, non-invasive brain stimulation (NIBS) approaches have rapidly progressed and become an unique approach for the causal exploration of brain-behavioural associations. For the last 4 decades, both TMS and tCS have enriched our understanding of human cognitive neuroanatomy and facilitated interesting insight in the physiological mechanisms CNS condition Furthermore, thanks to its ability to assess regional excitability and quantify local brain states, measure central conduction time across intracortical/ cortico-cortical/ cortico-spinal pathways they have proven valuable in diagnostic applications. Additionally, by leveraging its capacity to facilitate or suppress neural plasticity over time, continuous and repetitive stimulation patterns delivered across multiple days have been employed to actively or indirectly engage activity-dependent mechanisms, mediating the recovery of impaired functions.

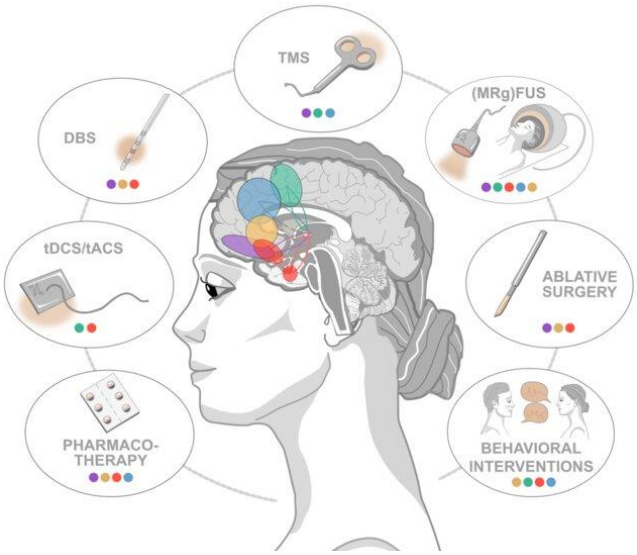


Figure 11. Brain stimulation neuromodulation options. Multiple options, strategies and tools are available for brain stimulation in experimental and clinical applications either invasive or transcranially. Pharmacological agents, electrical direct, alternating currents, with low or high current (electroconvulsive) stimulation, deep brain stimulation implants, magnetic stimulation, ultrasound stimulation, surgery, neurofeedback or behavioural interventions (Adapted from[139]).

Currently, multiple technologies are available for assessing causally the anatomical and neurophysiological correlates of cognition and neuromodulation. Invasive methods such surgical ablations, intracranial or deep brain stimulation compared to non-invasive methods such transcranial electrical or magnetic stimulation or more recently focused

ultrasound, differed in their own physiological mechanisms of action, temporal and spatial resolution, depth of action, set-up parameters, magnitude, duration and global level of efficacy. However, they all converge in a common overarching goal which is to achieve a modulation of human brain regions and networks that prove efficient, consistent, brain-informative and eventually clinically useful.

Since the advent and explosion of high resolution MRI and high density EEG based approaches, brain circuits have been characterized by studying their functional and structural correlates with fMRI and diffusion imaging (DWI) or their dynamics with EEG and MEG. Nonetheless, lacking causal power, neither neuroimaging nor neurophysiological correlates do automatically translate into effective targets for treating symptom or diseases because (i.e., temporal activation, regions involved, etc.) they may but also they may not have real bearings into a symptom be epiphenomenal, spurious, or compensatory. In an attempt to fill this 'causality gap'[140], approaches such as lesion network symptom mapping or retrospective machine learning (ML) to causal neuroimaging sources of information have been proposed. For example, circuits involved in a particular symptom or behaviour can be derived from retrospective analyses using brain stimulation clinical datasets, as there is a presumed causal link between the stimulation of target, its associated network and behaviour changes. As otherwise mentioned in the first section of this introduction, brain lesions provide a model to study causal involved circuits. Multiple studies have identified lesion-based neuroanatomical substrates of dysfunctional behaviors and generate a growing library of potential cortical or cortico-subcortical targets awaiting NIBS clinical trials. However, a key limitation of group-level regression analyses is that they do not account for individual variability in brain structure and function; instead, they highlight potential targets and circuits based on normative group models. Therefore, effective predictive, and beneficial non-invasive stimulation requires a process of personalization, optimizing and tailoring parameters and target locations to individual participants[141].

In that context, spatial localization of stimulation can be optimized based on anatomical space coordinates derived from individual structural MRI scans, on resting-state or task-based fMRI (BOLD activity) to maximize engagement of a functional circuit, or DWI individual tracts to maximize engagement of anatomically interconnected areas. Likewise, the underlying brain/network state of activity it is known to largely influence target engagement and stimulation effects. Electrophysiological signals from scalp recordings (EEG), intracranial (iEEG or SEEG) or epidural grids can be used to optimize timing and/or frequency parameters maximizing target engagement in 'open-loop' or 'close-loop' approaches (please see section on Combining NIBS with neuroimaging for more details). Similarly, task environments can be established to promote equivalent baseline activity states across individuals within a cohort and reduce variability, or manipulated to maximize a pre-engagement of certain brain networks to favour sensitivity to response[142] (Figure 12). Both, psychological rehabilitation and pharmacological conditioning can be thought as interventions employed to control baseline system state or potentiate outcomes via an effect of brain activity states[141]. Nonetheless, despite existing options for tracking circuits and maladaptive patterns of neural activity, no clear systematic approach has yet been stablished. It is hence plausible that a target may be identified using lesion network localization, then it may be personalized using DWI and electric field (E-field) modeling and paired with a psychological task; whereas alternative approaches for behavioral targets may

encompass identification using retrospective analyses of stimulated patient's cohorts, fMRI task engaged individualized spot, augmented with pharmacology and guided with scalp electrophysiological correlates[141,143–146].

The list of non-invasive tools that can be used for human neuromodulation has been expanding recently from Transcranial Magnetic Stimulation (TMS) and transcranial current electrical stimulation (tCS) in its sub-modalities (tACS, tACS and tRNS) delivering respectively direct, alternate or random noise current, to transcranial Time interference Stimulation (tTIS) and transcranial Focal Ultrasound Stimulation (tFUS), each one with its own mechanisms of action and advantages (Figure 11). For example, there were repetitive or rhythmic TMS may benefit from a well-known suprathreshold ability, high temporal and spatial resolution and a contrasted safety profile with flexibility of application in exploratory, diagnostic and therapeutic applications, tTUS may benefit from ultra-high spatial resolution and ability to target deep brain areas. Others like transcranial current electrical stimulation (tCS) is characterized for a unique safety profiles and easy implementation in clinical trials but poor spatial and temporal resolution, whereas tTIS could stimulate cortical or superficial areas with a directionally steerable current along a given axis without moving electrodes. Globally, given long-lasting experience in their use. tCS and TMS are most commonly employed for exploratory causal studies disorders such as major depression, stroke chronic pain, whereas thus far tTIS and tTUS have been less explored for research and clinical applications. Finally, a high intensity focused ultrasound (Hi-FUS) can induce permanent thermal lesions with but irreversible circuit changes and behavioural modulations.

Current electric (tCS) and magnetic (TMS) transcranial stimulation are the most commonly employed technologies in the field of neuromodulation. Electric stimulation is subthreshold (i.e., unable to trigger action potentials but to modulate resting membrane potential) technique that uses weak electrical fields (in humans 1-4mA, 0.1-0.6V/m) applied through electrodes on the scalp. Electrical current affects the polarization of neuronal membrane, altering neuronal excitability and synaptic efficiency generating and/or facilitating stochastic resonance and temporally biasing spike generation. The effectiveness of tCS depends on various experimental factors, including brain state and the magnitude of the electric field, which is influenced by the skull and the scalp high resistance (or low conductivity/permittivity). On the other side, TMS is a suprathreshold technique that uses time-varying high-voltage magnetic fields (100-200V/m) to generate high-voltage intracranial electrical currents in conductive tissues. TMS is widely used for research purposes to assess functional mapping, brain connectivity, excitability, plasticity and used for clinical applications. It triggers action potentials by depolarizing primarily pyramidal cell axons via an indirect effect via intracortical interneurons, leading to action potentials that propagate across axons and travel trans-synaptically networks. Depending on TMS pulse parameters organized in short burst or trains, TMS can either increase or decrease cortical excitability. Different protocols exist, including single-pulse, paired-pulse, and repetitive TMS (rTMS). Single-pulse TMS is used diagnostically to perturb "*online"* and to quantify brain states, whereas rTMS, delivering bursts of pulses over time modulates regional and associated network activity, beyond pulse/train/regime duration (so called *"offline" or "after-effects")* and by virtue of such, it has been used for its therapeutic potential. The effects of short TMS bursts at a given frequency, referred to as rhythmic TMS, have been also classified as related to the entrainment of frequency specific EEG rhythms at the delivered pulse rate. Nonetheless, whether and how this

directly affects excitability and synaptic plasticity remains to be clarified (please see General Methods for tDCS and TMS details).

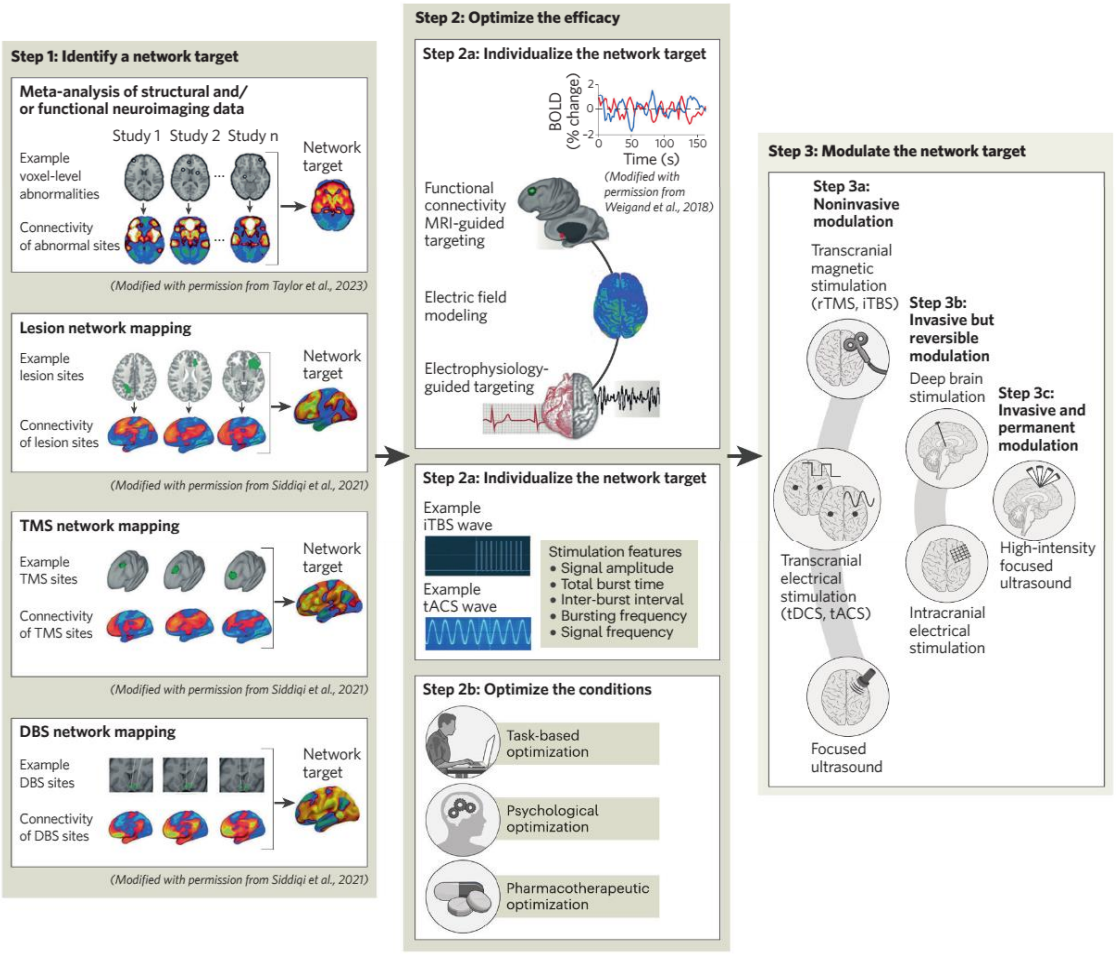


Figure 12. Diagram of personalized NIBS for experimental and clinical neuroscience. The development of personalized therapeutics in neuromodulation follows a structured process, addressing the critical questions of *where*, *when*, and *how* to intervene in order to modulate brain plasticity. This begins with the formulation of detailed pathophysiological models encompassing brain metabolism, structural abnormalities, and functional disorders to guide potential interventions. Causal neuroimaging plays a pivotal role in this stage, enabling retrospective meta-analyses that map symptoms to neural networks and decode maladaptive spatiotemporal communication patterns across brain regions. These insights help identify optimal targets for neuromodulation. The next step involves accounting for significant inter-individual variability that can influence target engagement and treatment efficacy. This optimization process leverages tools such as individual functional neuroimaging, computational modeling, and electrophysiological analyses to fine-tune spatial and temporal engagement with specific neural circuits. Moreover, dose-response effects can be calibrated by adjusting stimulation parameters to selectively activate desired neural processes. Contextual and environmental factors must also considered, with strategies such as pharmacological/behavioral priming employed to enhance the subject's responsiveness and improve outcomes. Finally, NIBS clinical application requires proof of short-term, causally beneficial neuromodulation, which serves as a foundation for the development of therapeutic interventions with long-lasting effects (Adapted from[141]).

1.2.1 Combining NIBS with neuroimaging

Over the last three decades, advances in mapping techniques have notably enriched human neuroscience research, providing sensitive tools and methods to identify brain regions in which correlates of neuronal activity (M/EEG amplitude, power, synchronization measure, or fMRI BOLD signal) correlates with behavioural outcomes. Although brain mapping can identify the spatial extent and the temporal profile of brain activation during an experimental task, brain imaging is correlational in nature. **In contrast, non-invasive brain stimulation contributes to the causal neuroscientific exploration of the CNS, by revealing the contribution of a cortical region (and associated network) and its locally occurring activity patters to a particular brain process (i.e. either motor, sensory or high level cognition)**, in a similar manner as classical local lesion studies would do.

Objective TMS-elicited Motor evoked potentials (MEPs), subjectively reported visual phosphenes (Phos) or quantitative effects on performance measures have been used to explore how NIBS modulates the human cortex. For many years, knowledge about the physiologic mechanisms of action for TMS was gathered studying the primary motor hand area (M1-HAND) in studies that recorded MEPs from an intrinsic hand muscle (first dorsal interosseus FDI or Abductor Pollicis Brevis APB). Other studies examined the behavioural consequences of TMS in well-defined experimental tasks. Although these initial studies highlighted the causal involvement of cortical areas in on M1 and premotor regions on execution and control, and addressed local M1 and corticospinal long lasting excitatory and inhibitory effects, they provided limited information regarding the underlying physiologic mechanisms. This motivated the concurrent use of neuroimaging techniques to map the acute ("*online*") and conditioning ("*offline*") effects of NIBS on brain function with two main aims: first, to explore the mechanisms underlying stimulation, and second, to inform stimulation providing information about -when -where and -how to stimulate to increment target engagement[147].

Over the last decade, successful readouts of NIBS effects on neuronal/regional activity have been extracted principally from the concurrent ("*online*") or sequential ("*offline*") combination of rTMS and tES with functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS) sensitive to blood oxygen level dependent (BOLD) or oxygenated hemoglobine (Hb02) signal, slow and delayed (~several seconds) relative to the neural activity; also position emission tomography (PET) and magnetic resonance spectroscopy (MRS) sensitive to metabolic (FDG, L-DOPA etc) and biochemical (GABA, changes at the seconds-minutes scale and electroencephalography (EEG) and Magnetoencephalography (MEG) sensitive to very fast (milliseconds) neuronal activity with poor spatial resolution (please see[148] for a review). The temporal and spatial resolution of neuroimaging techniques represent an essential selection criteria when planning a combined NIBS-mapping study. If temporal aspects of neuronal processing are the focus of interest, the use of a neurophysiological mapping method of high temporal resolution such as high-density EEG will be preferable to any hemodynamic fMRI based approach. Conversely, if the study goal of the experiment is to assess precisely the spatial pattern of TMS-tES induced changes in brain activity, a neuroimaging method with superior spatial resolution such as fMRI will be adopted (Figure 13).

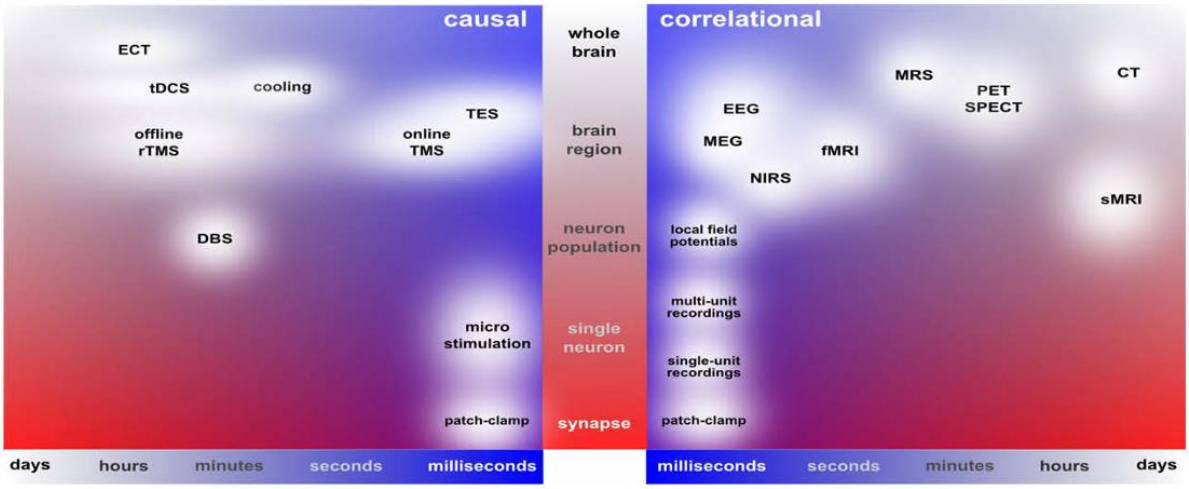


Figure 13. Temporal-spatial resolution of neuroimaging/neurophysiological (correlational) and stimulation (causal) methods to study brain function (Adapted from[147]).

Another important factor to consider is the timing at which to carry out the recordings needed to address the research questions. Neuroimaging or neurophysiological readouts can be obtained concurrently ("*offline*") with the delivery of NIBS, offering indices or measurements of causal changes on neural activity normally performed after a reversible plasticity-inducing protocol to capture neuromodulator after-effects that outlast from minutes to hours the duration of the pattern, or concurrently ("*online*") offering insights into effects arising during or immediately after NIBS. Both strategies can be further employed to develop precise brain-informed (anatomy and physiology) stimulation in a "close-loop" manner.

One way to capture the immediate effects of NIBS on regional brain activity is to perform recordings simultaneously with the stimulation. Depending on the applied technique and the stimulation parameters, "online" NIBS can be used to quantify local activity by applying stimuli strong enough to evoke a direct output (i.e., suprathreshold), to interfere or perturb ongoing spontaneous activity related to a task the participants is performing, or to modulate or entrain the performance level or the timing of spontaneous or task-evoked activity. The interpretation of "*online*" neuroimaging studies can be complex because TMS and tES results in multimodal sensory stimulation together with synaptic activity. Therefore, proper active and sham controls are needed to rule out neural activations driven by peripheral confounding factors in our data. Another way of detecting the conditioning effects of NIBS on regional neuronal activity is to compare before and after the delivery of the NIBS pattern. This "*offline*" approach applies conditioning reversible protocols that may result in net terms in long-term potentiation (LTP)-like or long-term depression (LTD)-like plasticity effects. Neuroimaging/ neurophysiological approaches should onset as quickly as possible after the end of TMS or tCS to ensure that short-lasting aftereffects are captured. However, any change in activation may simply be a time epiphenomenal effect. To dissociate temporal order effects from 'active' effects, the experimental designs should include a sham and/or active controls. Both *online* and *offline* neuroimaging/neurophysiological readouts can be used to inform stimulation and to individualize stimulation location, parameters and timing. As mentioned before, cortical normative targets need to be individualized, and target site localized in each subject structurally and functionally[149]. The position and orientation of the stimulation devices, such as TMS coils of tCS electrodes can be optimized according to individual anatomical markers extracted from structural scans,

based on neuroanatomical landmarks, defined from the individual parcellation of functional brain networks or structural white matter pathways or source-localization of task-engaged activation (Figure 14). For precise experimental placement, MRI-based neuronavigational procedures with those predefined targets have gained outstanding popularity in the last 10 years.

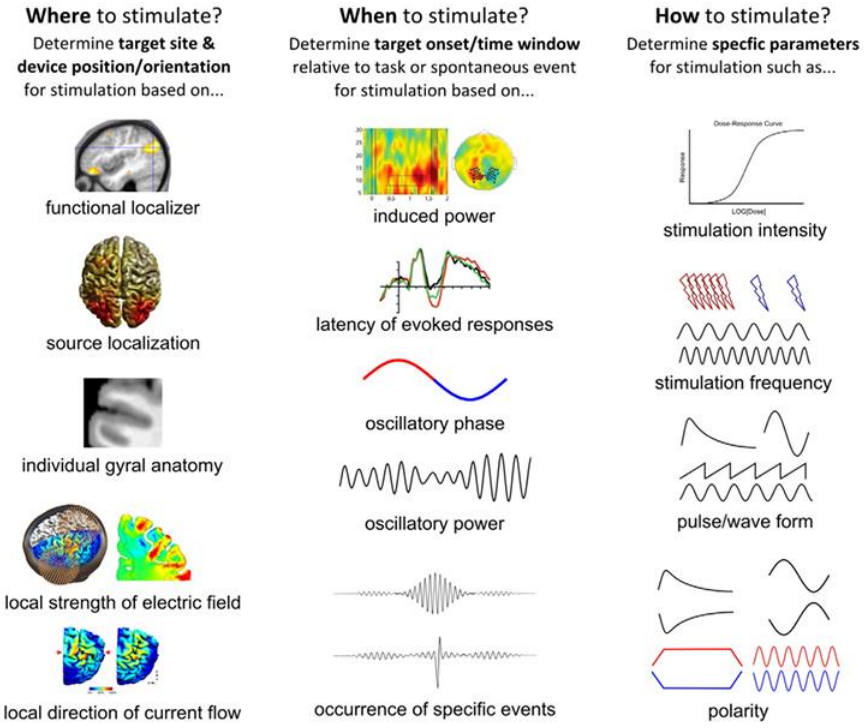


Figure 14. Neuroimaging-informed NIBS. Integrating structural and functional brain measurements enhances the spatiotemporal precision of NIBS interventions and guides the spatial, temporal, and parametric design of individualized stimulation protocols. Structural or functional MRI can be utilized to precisely localize cortical targets, informing the placement of TMS coils or tCS electrodes to direct electric fields towards regions of interest. Electric field simulations further refine this process by estimating target engagement and predicting the macroscopic regional or microscopic cellular depolarization effects. Beyond spatial precision, temporal optimization is critical, as brain states and cortical excitability fluctuate rapidly. Identifying the optimal target onset relative to the underlying brain state is essential for predicting neural effects. Stimulation timings can be aligned with periodic or aperiodic processes, as well as synchronization events, to maximize efficacy. Finally, stimulation parameters, including intensity, frequency or pulse shape, play a crucial role in modulating neural responses and determining which neuronal populations are primarily engaged. Parameters are habitually predefined based on previous findings, but also subtended to some degree of personalization. For example, stimulation intensity can be adjusted to individual motor thresholds, and frequency can be adapted to individual predominant natural frequency. Altogether, these neuroimaging-informed approaches enable more precise, personalized, and effective non-invasive brain stimulation strategies (Adapted from[148]).

Furthermore, precise activation requires the prediction and optimization of TMS coil size and shape, or electrodes placement based on the distribution of the electric fields delivered[148]. Optimization algorithms and open-access software (e.g., SIMNIBS, ROAST) have become quite mainstream in for these purposes but total customization, subject-by-subject or patient-by-patient, more crucial for tCS than TMS is rarely practiced. In parallel, further research is needed to demonstrate improvement of NIIBS

efficacy when using advanced versus traditional targeting strategies. Stimulation timing is essential as demonstrated for input-output gain and neuronal communication physiology. For that reason, NIBS techniques are combined with highly temporally precise mapping technics, particularly EEG[146]. Precise timing is also paramount in causal experimental neuroscience because it allows to quantify in a temporally specific manner the role of ongoing local and network dynamics in interaction with the own temporal dynamics of the modulation source. ¨*Online*¨ *TMS* triggering based on a feature or features of ongoing EEG (phase, amplitude, power) is only feasible when the signal-to-noise ratio is sufficiently high. However, this only applies to ongoing neuronal oscillations or highly synchronized neural events when power and even instantaneous amplitude and phase can be reliably estimated online. Finally, parameters of stimulation can also be adjusted from brain structural priors. For example, the individually-adjusted stimulation intensity necessary for evoking neural activity can be anticipated from the amplitude of Transcranial Evoked Potentials, combing TMS with EEG, or the amplitude of MEPs combing TMS with EMG[150]. Scalp-cortex distance can be derived from structural MRI data and stimulation intensity can be adjusted accordingly.

2. CHALLENGES AND AIMS

2.1. General aims

The long-term goal of the project is to further develop the use from weak to conventional intensity E-fields generated by non-invasive brain stimulation concurrently with mapping techniques to causally characterize and modulate neural processing driven by excitability modes or oscillations in health and disease. The project aims at developing, conceptual, technical, experimental and clinical expertise that helps pioneering innovative networked NIBS approaches suited to manipulate extended networks.

The short-term goal of this thesis is to (1) To enhance reliability in NIBS-EEG analyses by refining TMS-EEG analysis methods based on spatial filtering signal bases on biophysical E-field distribution models; Also, (2) to use such tools to explore the physiological effects of very-weak to conventional intensity TMS on the primary motor cortex and uncover subtle, underexplored neural effects; Finally, (3) To evaluate the clinical potential of tDCS neuromodulation for targeted, large-scale network engagement.

2.2. Specific aims

2.2.1 Specific aim one – Thesis Experimental section 1

Specific Aim 1: We will develop a novel technical framework and tools for causal neuroimaging, enabling researchers to enhance the reliability and spatial resolution of causal TMS-EEG biomarkers.

Prior evidence has shown that sustained or episodic oscillations and synchronization phenomena have a role in the emergence of cognitive operations, by enabling neural processing and information transfer. Moreover, dysfunctional oscillatory activity has been causally associated with neurological symptoms, hence, by developing novel sensitive TMS-EEG tools improving the spatial specificity of neurophysiological biomarkers, we will better inform hence personalize NIBS rehabilitation strategies.

Hypothesis 1: We hypothesized (Experimental section 1), that E-field modelling will provide useful computational priors from which to extract EEG biomarkers of neural activity in NIBS-EEG settings, which will improve the reliability of biomarker detection to characterize pathological neural function and improve NIBS targeting, engagement and efficacy.

Challenge 1: Several technical challenges are posed by the low spatial resolution and low specificity of scalp EEG recordings, the intrusiveness and duration of electrical artefacts induced by NIBS on EEG readouts employed to monitor TMS effects, the large individual variability in human brain topology and the linear superimposition (multiplicity of sources involved in the signal generation).

Deliverable 1: The combination of TMS E-field SIMNIBS 4.0 modelling with spatial EEG filtering procedures will be employed to develop a new computational framework and tool to extract neural EEG signatures of local evoked source activity.

2.2.2 Specific aim two – Thesis Experimental section 2

Specific Aim 2: We will evaluate the impact of very weak TMS entrainment trains to establish a physiological framework addressing the relationship between magnetic field intensity and elicited oscillatory activity.

Transcranial magnetic stimulation has become mainstream for the causal exploration of brain function and for clinical neuromodulation. However the effects of very weak intensity pulses and patterns and differential subtending effects compared to conventional high intensity TMS remain debated and the mechanisms of action still not completely understood. Human TMS in M1 has been classically indexed to a physiological response such as MEP threshold requiring high levels of current strength. Nonetheless, rodent work has shown that very weak TMS can evoke neural activity and induce long-lasting inhibition. In 3 consecutive experiments, conventional TMS-EEG approaches delivering single pulses, short 20 Hz bursts or long 10 Hz trains at very weak (compared to conventional intensities) we will assess impact on (1) TEPs and oscillatory measures of entrainment; (2) oscillatory input-output recruitment at increasing levels of subthreshold intensity and finally, (3) Offline repetitive TMS.

Hypothesis 2: We hypothesize (Experimental section 2) that very weak magnetic fields will show a significant impact on neuronal function by selectively modulating neocortical oscillatory activity or excitability, suggesting its suitability for human rTMS neuromodulation.

Challenge 2: The main challenge for this aim is posed by our incomplete understanding on the potential mechanisms by which very weak magnetic fields (TMS) could impact the human brain and the sensibility of our techniques.

Deliverable 2: A procedure and dataset using very weak transcranial magnetic stimulation combined with concurrent EEG and EMG to characterize effects of very weak 20 Hz TMS on direct "*online*" neural oscillatory signatures, and post rTMS "offline" signatures, suggestive of short-term plasticity.

2.2.3 Specific aim three – Thesis Experimental section 3

Specific Aim 3: We will provide evidence that the isolated or combined stimulation with anodal tDCS of non-purely motor brain cortical networks with indirect bearing on motor function has the ability to drive motor recovery.

Middle cerebral artery (MCA) strokes cause structural damage to key sensory-motor networks in charge of controlling and executing voluntary movements, however the magnitude of deficits has to be explained by both direct but also diaschisis effects altering network communication with structures contributing to optimal motor execution.

Here, we will specifically target during two weeks of an anodal high density tDCS montage the ipsilesional dorsolateral prefrontal cortex (DLPFC) involved in cognitive control, sustained attention, reward and motivation and planning and the contralesional cerebellum contributing to visuomotor learning and adaptation. We aim to promote activity increases and adaptive through salient residual functional. Our trial positions the subject in the center of the research exploring direct functional voluntary changes, but equally important, serves as a physiological model to characterize the anatomical, neurophysiological and behavioural contributions of frontal and cerebellar regions to the modulation of voluntary motion.

Hypothesis 3: We hypothesize that (Experimental section 3) multifocal tDCS non-invasive brain stimulation will prove a well-tolerated useful strategy to boost sensorimotor functions and promote adaptive plastic reorganization in post-stroke patients by activating pre-existing spared areas and connections affected by diaschisis.

Challenge 3: We hypothesize the excellent feasibility and tolerability of the protocol and a limited efficacy of both single-region anodal tDCS stimulation conditions compared to a double site combined strategy to improve motor deficits.

Deliverable 3: A optimized electric fields based tDCS protocol and strategy to explore the neuromodulation potential and limitations of a novel dual-site high density weak intensity stimulation project improving motor performance following post-stroke damage.

3. GENERAL METHODS

The present dissertation is the result of three independent staggered experimental sections combining three categories of methodological approaches. For all studies, experimental design and methods varied according to the research questions and objectives. *Experimental section 1* encompasses a technical/methodological section in which we designed and implemented a novel procedure for TMS-EEG signal reconstruction, introducing an electric-field (E-field) derived spatial filter. To this end, computational biophysical modelling and linear estimators for source EEG reconstruction were employed. In *Experimental section 2,* we evaluated the impact of weak TMS intensity pattens with three consecutive experiments combining TMS-EEG and EMG recordings, in which we evaluated '*online'* direct modulatory effects via estimates of local oscillatory activity entrained with short 20 Hz bursts, cortical reactivity via transcranial evoked potentials and oscillatory measurements within an input-output curve-like framework at different levels of subthreshold stimulation and finally also '*offline'* 10 Hz rTMS after-effects on MEP amplitude and paired-pulse intracortical excitability paradigms. Finally, in *Experimental section 3*, we evaluated experimentally in a cohort of motor stroke patients a multifocal anodal tDCS strategy, targeting either the ipsilesional dorsolateral prefrontal cortex, the contralesional anterior lobe of the cerebellum, or the combination thereof by means of clinically-based motor evaluations.

In this General Methods section, we explain for those that might not be necessarily familiar with such, some general aspects of the methods employed in our studies, emphasizing their potential and limitations and sharing with the reader some of the challenges we went through to adapt them to human participants and patients. Non-invasive stimulation methods employed are detailed (TMS, tDCS), followed by the main mapping method we used (electroencephalography, EEG) and its combination with TMS. Next, statistical procedures used along the studies (classical statistics and cluster-based permutation analyses for EEG data) are introduced, computational modelling theory and procedures (E-field modelling) are summarized, and finally, an overview of the motor functional evaluations (clinical and experimental) are presented.

3.1. Transcranial Magnetic Stimulation: TMS

Transcranial magnetic stimulation is a non-invasive method for brain stimulation first introduced and reported by Barker and colleagues (1985)[151] based on the magnetic induction principles discovered by Faraday in the first half of the 19th century. A brief short-lasting (100-300μs) high intensity (i.e., 7-15 mA·cm^2, 100-200V/m) electrical current is passed through a loop of conducting wire loops (copper for most TMS commercially available devices) placed inside a protective insulated plastic case (aka. TMS Coil), generating a strong time-varying electromagnetic field (E-field) perpendicular to the transducing coil[152]. The current generated by the magnetic pulsed field, penetrates through the skin and the cranium and progresses with a strength that attenuates as a function of distance and tissue thickness to end up inducing a phasic electric current in excitable tissues[153–157].

To be able to deliver a pulse or a train of pulses, TMS equipment consists of three main components: A stimulation coil containing loops of copper wire to generate the magnetic field; a central unit to set up the amount of current and synchronize its release at a given time; finally, a group of capacitors to accumulate high loads of electric charge[158]. A central control unit (often interfaced with a computer) is wired to a set of

capacitors filled with high voltage current from power lines. From the central unit one can set the different stimulation parameters, such as, pulse/pattern intensity indicated as a relative percentage of the maximum stimulator output (of current level/density of induced charge in mV/m2) and the pattern frequency (measured in Hertz). The 'charge-discharge' control unit, and discharges current according to intensity, number of pulses and burst/pattern frequency settings, via a TMS stimulation coil placed on the participant's head. The latter is a key component of the equipment since it transfers the magnetic field energy to the neuronal tissue via a perpendicular penetrating electrical current and determines the shape and penetration capacity of the ultimately induced electric current parallel to the coil-surface[159–161]. In the current doctoral work, all TMS experiments used a figure-of-eight TMS coil (also referred to as double or butterfly shaped coils), made of two side-by-side round coils having with a diameter of 25-70 mm, so that the electrical field under their junction flows in opposite directions, ensuring a more focal (1.5-2 cm^2) field where the two circles meet. Maximal intensity of the TMS-induced E-field will depend on the capacitator- and coil- capability combinations, numerically estimated from Faraday's law.

Applied perpendicularly onto cortical areas, **TMS electrical field (E-field) will directly operate on the level of the resting membrane potential in underlying neural assemblies according to their spatial distribution and excitability state.** If the magnitude of the electric field induced by the TMS pulse is sufficiently strong to depolarize at a given time and under existing conditions the resting membrane potential of a given neuron and bring it above its firing threshold, an action potential will be triggered[13,54]. When applied onto the primary motor cortex (M1) at the so-called supra-motor threshold intensities, single pulses or short bursts of TMS pulses can activate the fibres of the corticospinal tract, inducing twitches in upper limb or lower limb muscles associated with M1 cortical representation being reached by the stimulation. If applied to the occipital pole, these same TMS patterns can elicit a 'phosphene' visual percept that will change in position depending on the exact coil position following the retinotopic organization of the primary visual areas (V1 to V4). When applied to non-motor or non-visual cortical regions to nodes of networks subtending higher cognitive functions (language, memory, attention, etc.) without a directly measurable objective physiological responses, TMS disrupts/boost/modulates encoding rhythms and interferes physiological and/or behavioural activities mediated by the targeted cortical regions. Importantly TMS has not only local but distant effects conveyed through connectivity, where TMS primary cortical depolarization is spread across directly interconnected structures and indirectly to other networks depending on the wild nature of the connections targeted. Thus, TMS can facilitate cortical excitability increases in a determined cortical region (area A), which may drive in a similar manner and power, inhibition in another region (area B) if the net sign of the connectivity between these two regions is dominantly inhibitory (stimulation and increase of activity in area A-> inhibitory projections -> inhibition area B)[162,163] (Figure 15). It is thought that TMS directly stimulates neurons through depolarization of myelinated axons, primarily depolarizing projection terminals, changes in diameter and bends (e.g., corticofugal axons curving into subcortical white matter), primary located in the crown and lips of the neocortex[164].

TMS primary cortical currents are capable to initiate action potentials which will propagate *orthodromically* (from neuronal bodies to axon terminals) and *antidromically* (from axon terminals to neuronal bodies and back) simultaneously from A-to-B and also from B-to-A, inducing downstream and upstream synaptic transmission, eventually generating complex patterns re-entry/reverberative activity as time post pulse is let to evolve. The local engagement and network spread of TMS activity will be highly

influenced by neuronal threshold levels, the directional sensitivity of neurons and axons with regards to the generated magnetic fields[165], and fluctuating brain excitability states.

Building on such mechanisms, TMS has carved a niche of its own in the domain of neuromodulation for the following activities: (1) To establish causality between structural sites and patterns of functional activity; (2) To causality probe interactions of a functional brain state and a high order cognitive process or behavioural contribution; (3) To explore and characterize local and network pathological brain states in disease; (4) To guide and boost plasticity processes proven adaptive for the recovery after brain damage.

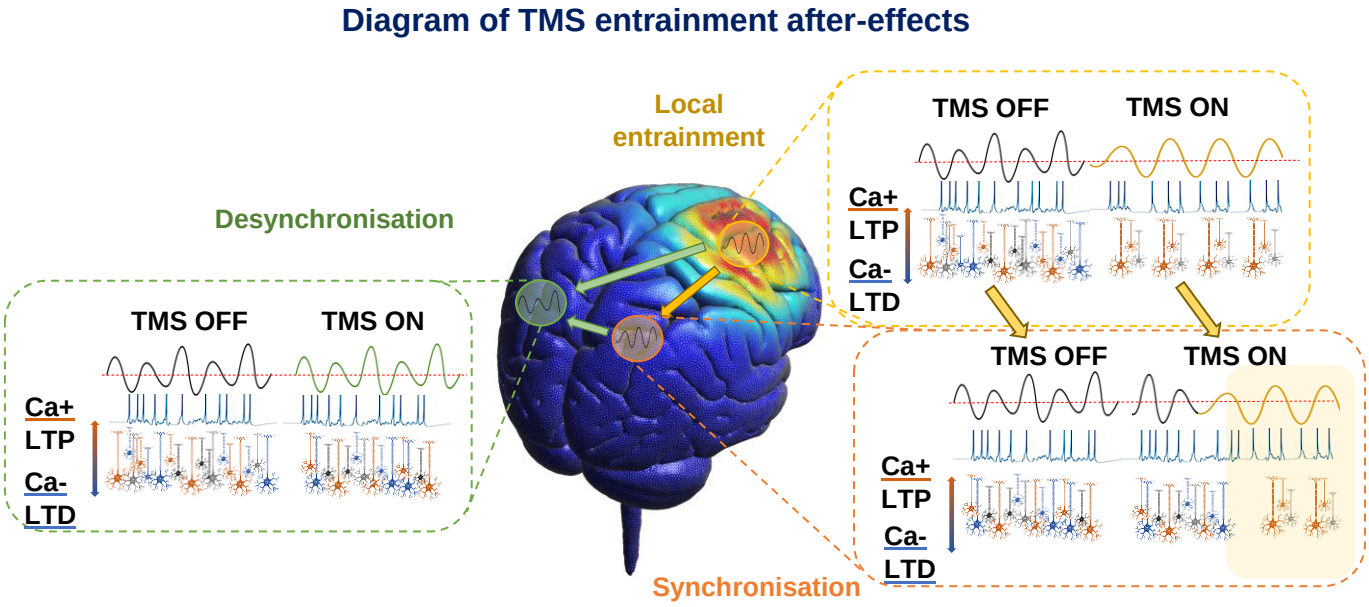


Figure 15. Pictural mode to exemplify the impact of TMS entrainment in brain activity. Source oscillators have their own temporal dynamics and natural oscillatory frequency. A periodic external force exerted by series of single TMS pulses repeated rhythmically at a given frequency, phase resets the cycles of the targeted local neural assemblies, hence progressively phase-locking (i.e., synchronizing) their rhythms and activity. This local changes induce the synchronization or desynchronization of long distant connected populations and oscillators according to the underlying circuit characteristics[162]. TMS intensities set at supra-action potential threshold may impose a frequency specific rhythm according to TMS frequency, however TMS sub-action potential threshold induce polarity shifts capable of entraining endogenous oscillators and modulate ongoing rhythms.

For that purpose, **multiple combinations of TMS parameters including pulse intensity (amplitude), pulse number, burst/pattern frequency, duration and TMS free interpulse/interburst interval, have been employed to either entrain activity, modulate, interfere or quantify**[148]**.** A series of single pulse TMS consists in the generation of isolated magnetic field waves (aka monophasic or biphasic pulses) followed by long time intervals (between 4 to 8 seconds) so that their individual effects do not sum up over time and generate carry over effects. Single pulses can be used for diagnostic purposes to estimate the degree of residual corticospinal structural/functional conduction and its excitability or to probe fundamental physiological properties of cortical regions and networks, such as like ‘input-output gain’ or ‘pulsed facilitation-inhibition[166]. Double pulse or paired Pulse TMS consists in the discharge of a test stimulus (TS) preceded by a conditioning stimulus (CS) delayed by an interstimulus

interval (ISI). They have been mainly employed to assess local primary motor system (M1) intracortical modulatory mechanisms (SICI, IFC, etc.) or cortico-cortical interregional interactions between two regions (please see 1.1.4 Input-output models of human plasticity section for more details and physiological relevance of paired pulse parameters combination)[55]. Finally, repetitive TMS (rTMS) refers to any combination of more than two pulses, and long periods of stimulation (up to 20-30 minutes) at a fixed frequency, with or without interruption by stimulation-free intervals in between discharges with an ability to produce different effects than the isolated pulses. Largely used in experimental scenarios and also pre-therapeutic/therapeutic settings, and according to Hebbian plasticity and early LTD-LTP experiments, rTMS has shown to induce long lasting excitation (principally with higher frequency trains like 20Hz or intermittent theta burst) or inhibition (principally with lower frequency trains like 1Hz or continuous theta burst). Despite repetitive TMS has been largely considered and often described as LTP or LTD-like since the duration of cortical excitability changes last over time (aftereffects) is dependent of the length of simulation, nonetheless, their more detailed relation with synaptic plasticity remains elusive. Classical repetitive excitatory (>5Hz and intermittent Theta Burst Stimulation, iTBS, triplets of 50 Hz repeated at 5 Hz) and inhibitory (= or < 1 Hz repetitive stimulation, and continuous Theta Burst Stimulation, iTBS, triplets of 50 Hz repeated at 5 Hz) have shown increases or decreases of EEG oscillatory power, changes in fMRI BOLD signal and changes of MEP excitability. However whether different rTMS protocols truly operates at the synaptic level also remains to be confirmed[77,167,168]. In the present dissertation, *Experimental section 2* includes 3 different experiments which employed different patterns of TMS stimuli: (1) The 1st experiment employed single pulses to evoke activity and short 20Hz rhythmic bursts to entrain (phase synchronizing local oscillators) in the primary motor cortex; (2) in the 2nd experiment, rhythmic repetitive 20Hz short bursts were employed to entrain activity; finally (3) in the 3rd study repetitive 10Hz TMS for 28 minutes was employed, preceded and followed by single pulse and paired pulse metrics to quantify brain excitability.

3.2. Transcranial electrical stimulation: tDCS

Transcranial Electrical stimulation (tES) of the brain has a long history in science and medicine. Developed the middle of the XIX century (~1860), tES used brief high voltage currents applied via local scalp electrode for neuromodulation purposes. However, late 1990s the advent of TMS and later on the rediscovery (as it had been used briefly during the late 60s) of transcranial current stimulation (tCS), overcome its painful sensory effects and replaced tES. Transcranial direct current stimulation (tDCS) is among the most popular current-based non-invasive brain stimulation approaches used in experimental and mainly in clinical settings. It coexists with other types of current based patterns such as transcranial Alternate Current Stimulation (tACS) and transcranial Random Noise Stimulation (tRNS). It is characterized by its portability, low cost, ease of use, a safe side-effects profile and high flexibility to target with multiple channel systems, several locations simultaneously. **Transcranial DCS is based on the application of a continuous low-intensity electrical current inducing polarity-dependent sub-threshold shifts of resting membrane potential away (cathodal hyperpolarisation) or towards (anodal depolarization) from the neuronal firing thresholds**[12,75,169–173]. By virtue of such effects, single sessions of tDCS have shown the ability to

transiently modulate cortico-spinal excitability, whereas periodical sessions promote LTD-like and LTP-like plasticity.

Transcranial DCS is based on the application of weak electric fields (1-10mA) through sponge saline or gel-based electrodes, at low cortical current voltages below action potentials oscillating between 0.1 and 10 V/m. When applied to brain tissue, it affects the distribution of electrical charges (ions, Na+, K+, Cl-) between the extracellular and intracellular media, polarizing neuronal membranes, shifting the resting membrane potential towards or away from during threshold, altering neuronal excitability and spontaneous or stimulus-induced spiking. In addition, tonic depolarization of axon terminals can result in the release neurotransmitters vesicles in axon projection-specific manner altering synaptic efficiency, without necessarily triggering an action potential. However, the immediate *'online'* effects of tDCS stimulation have been shown to vary with the strength of the induced E-field, an observation that suggests that the effectiveness of stimulation and its consequences on neuronal local and network activity might depend on experimental variables, such as brain state. Experimental research *in silico* and *in culture* brain slices, in animal *in vivo* anesthetized or awake models, and also awake humans research work has been invested in characterizing the effects of tDCS on neuronal activity and establish dose-response relationships. **Depending on impact magnitude, five different mechanisms of action have been associated to its immediate online effects: *stochastic resonance*, *rhythm resonance*, *temporal biasing of neuronal spikes*, *entrainment of network patterns*, and *imposed patterns***[169,174–176]. The effects induced by such mechanisms can coexist, cooperate or compete with each other and they are confronted with endogenous brain activity. A critical point to be established prior to tDCS intervention is the magnitude of the electric field reaching the cortical surface with conventional tDCS. Applied at peak intensity currents in humans, tDCS current delivered at 1mA will generate maximal electric fields at the cortical surface under the electrodes of less than 0.5V/m. The main barrier for tDCS to reach the brain surface is the skull and the scalp, with high resistance (~160 Ωm) and low resistance (~2 Ωm) respectively. A large fraction of the current (up to 75%) is dissipated/shunted through and across the skin, becoming a source of 'peripheral' stimulation with an uncertain role on brain net responses[177–179]. Likewise, accidental activation of peripheral nerve terminals of the neck and head, cranial nerves and retinal cells by field spread may lead to neuromodulatory effects of their own on neural activity that study designs need to minimize and control for.

Unfortunately, due uncontrollable factors and additional putative mechanisms initiated during stimulation (activation of glial cells, vasodilation and neurovascular coupling, changes in blood-barrier permeability, etc.), the current state of knowledge on the physiological mechanisms of tCS remains limited and not exempt of controversy[180]. Moreover, despite classical models of tDCS stated that effects on neuronal excitability were dependent on the polarity of the electrodes, more recent investigations have suggested that it is the cytoarchitectural distribution of the neuroaxis perpendicular to the gyral and sulcal surface, relative to the direction of the electric field that plays a critical role[181,182]. To this regard, computational models have shown that different neuronal compartments have different transfer functions (i.e., sensitivity to the electrical E-field) so that depending on their relative orientation with regards to the electric fields, they may get hyperpolarized or depolarized. As a rule, cortical neurons with large cellular neuroaxis aligned from dendrites to cell bodies (notably, layer 5 pyramidal neocortical neurons) along the direction of the E-field will show increased probability of soma depolarization and therefore higher levels of synaptic spontaneous activity[183,184]. The parameters delivered by tDCS equipment (see, field intensity,

duration, current ramp-up and ramp-down time) are well-defined and consistently set, whereas the influence of other factors is harder to understand (e.g., individual tissue properties and head/brain anatomy features, age, gender, brain activity state before and during stimulation) or to control for. Due to the high individual variability of these factors electrical stimulation cannot be adequately dosed, hence the physiological/therapeutic outcome cannot be easily predicted or estimated. Consequently, large incongruences on the physiological effects of tDCS and uncertainty with regards to its ability to modulate cognition and behaviour are raised. Transcranial DCS electrodes and intensities can be arbitrarily set from a pre-computed standard head/brain model to ensure minimum voltage current levels in cortical or subcortical regions. For that purpose, optimization algorithms[185–187] can be used *a priori* in normative standardized models (such MNI152) under the 'risky' assumption of the lack of significant inter-individual variability in tissue volumes and thickness across participants. Nonetheless, individualisation of electrode positions and intensities has recently gained popularity as a tool to increase tDCS efficacy and ensure more consistent outcomes. This is achieved via a use of individualized MRI derived brain mesh reconstructions from which then position stimulation electrode positions or intensities ensure a minimum stimulation strength in the aimed brain target. Similarly, classical tDCS was administrated with 2 electrodes (one anode, one cathode) with relatively large surfaces (e.g., 5x7cm). Nowadays, small elliptic electrodes (e.g., 1cm^2) are commonly used with multiple simultaneous active cathodal or anodal electrodes to generate complex current spatial distributions according to each specific experimental or clinical need (Figure 16).

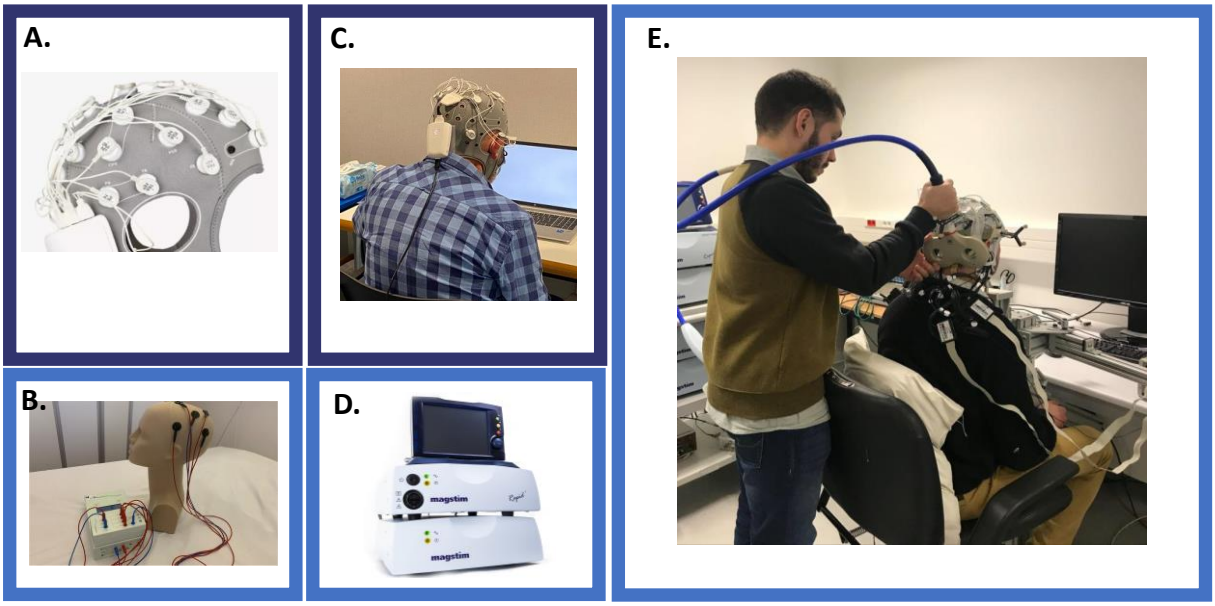


Figure 16. Methods: technical employed equipment for tDCS, TMS, and concurrent TMS-EEG sessions. A & B. Transcranial current stimulation (tCS) is delivered through a small portable battery-operated unit. Current is conveyed by short wires to an electrode montage (at least two electrodes) placed on the scalp based on 10/20 EEG reference system with sponge contacts, or solid ferromagnetic leads. Some systems (**A**) integrate EEG contacts for concurrent monitoring during stimulation and wireless control. **C.** The wireless tDCS/EEG device is mounted into a neoprene cap positioned in the head of a participant (*Experimental section 3* of this dissertation) performing a task in front of a computer screen. A weak electrical continuous current flows between an active (eventually several) 'anodal' electrode towards a single return 'cathode' placed apart to avoid current shunting, inducing on the brain surface a rather large polarization gradient modulating the resting membrane potential in the exposed neuronal clusters. **D.** TMS (employed in *Experimental sections* 1 & 2 of this dissertation) requires heavy non-portable and expensive equipment capable of depolarizing neuronal assemblies inducing action potentials in the brain. From a central control unit or CPU (**D**), stored electrical currents are circulated through a stimulation coil (in the picture **E.** a double figure-of-8 70 mm coil) to generate a brief magnetic pulse, capable of penetrating through skull tissue layers and induce a focally distributed electrical currents. Simultaneously, targeting is monitored via an MRI-based neuronavigation system. **E.** Concurrent TMS-EEG monitoring requires compatible scalp electrodes and amplifiers to avoid signal saturation by TMS artifact. EEG is capable of tracking neural activity milliseconds after the TMS charge allowing for precise scalp neural recordings of evoked or induced events.

3.3. Electroencephalography and signal processing: fundamentals

Scalp electroencephalography (EEG) is a correlational neurophysiological functional mapping method able to record neuronal activity transcranially, hence non-invasively. Its use is complex and a full characterisation of recording and analysis procedures allowing replication requires considerable attention and a detailed description of all steps followed. Nevertheless, the purpose of this section is to introduce EEG fundamentals and provide a general framework for our experimental conducted research in the scope of the present thesis. **EEG mostly reflects the summation of local excitatory and inhibitory postsynaptic currents or potentials (EPSC or IPSC) with millisecond temporal precision**. Because electrical conductivity through air is almost zero, a physical electrical bridge between the skin of the scalp and the electrode must be formed,

this is the reason that most EEG systems use conductive gel. The unit of measurement of EEG is volts (typically, microvolts or μV), however, microvolts are relative values such that the microvolt recorded from an electrode is the change in the measured electrical potential between that electrode and a reference electrode placed somewhere else on the head[188].

When neurotransmitters activate ion channels on cell membranes, electrically charged ions flow in and out of neurons, hence from and to the extracellular/intracellular space, causing a change of voltage. Post synaptic potentials are primarily confined to the dendrites and cell bodies. EEG signals can detect both radial and tangential sources (orientation of the activity dipole with respect to the skull) and is maximally sensitive to radial dipoles on gyral crowns (Figure 17). Then, recorded EEG activity emerges from the summation of voltage changes across thousands of neurons (i.e., across large field potentials), where local rhythmic fluctuations in the excitability of neurons or populations of neurons generate stimulus evoked or spontaneous evoked potential and patterns of oscillations, which can be measured transcranially. Unfortunately, EEG cannot measure individual molecular or synaptic events, nor can isolate events that are produced by a specific neurotransmitter or neuromodulator. EEG cannot measure neural polarization by a single neuron nor from very small ensembles of dozens of neurons despite small-scale events may encompass cortical layer-specific oscillations. Spatially specific small-scale events produce either no electrical field or weak electrical measurable only with

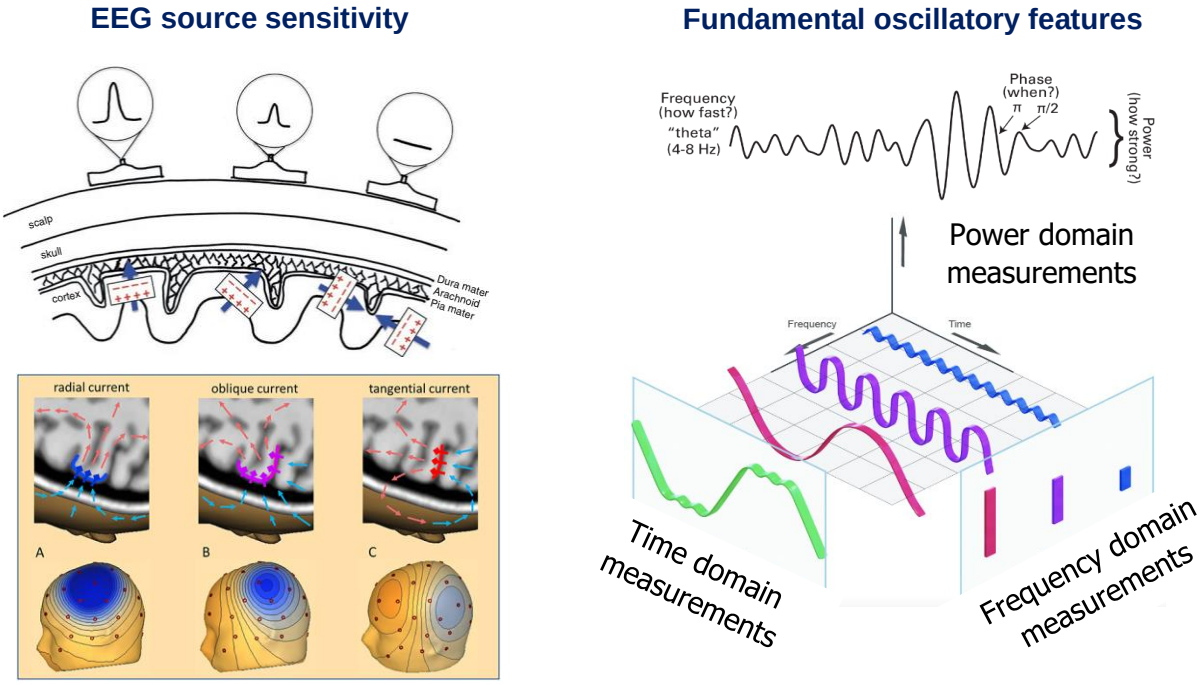


Figure 17. EEG source sensitivity and oscillatory features. Postsynaptic currents can be recorded extracranially using scalp sensors, provided that thousands of neurons synchronize their activity in common rhythms. EEG signals capture postsynaptic events originating from neuronal populations oriented in various directions, though they are most sensitive to radial dipoles generated by gyral crowns. Depending on the orientation of the source, distinct topographic maps reflecting voltage differences across sensors can reveal the brain region activated and the current's direction (left panel). EEG signals consist of both aperiodic and periodic components. Among these, evoked potentials and oscillations stand out as key features, representing neuronal communication and information transfer across populations. EEG oscillations are characterized by their frequency (cycles per second), amplitude (power of the evoked activity measured in μV), and phase (angular degree at a specific time point), all of which vary depending on the transient state of the brain (right panel).

invasive electrophysiological recordings. Nonetheless, activity from deep brain sources such as the thalamus, the basal ganglia, the hippocampus, and the brainstem are difficult but not impossible to record from the scalp. Oscillatory EEG patterns are mainly reflecting the interaction between large groups of interconnected neurons, hence essentially, network activity.

Neural oscillations (aka. periodical activity) are the most prominent feature of spontaneous EEG data (Figure 17). They arise from different biophysical mechanisms that depend on the intrinsic properties of local microcircuits affected by ion channels, connections, or extrinsic properties such as *input strength* and *noise levels*[24]. Oscillations exert complex spatiotemporal patterns that vary in amplitude, timing, phase and frequency. Neural oscillations are principally characterized by estimating the total number of cycles per second (aka. frequency, i.e., how fast electrical activity fluctuates periodically), by exploring the amplitude or power of the oscillation, and the phase, representing the transition between excitatory (peak or through) and inhibitory (peak or through) states and the degree of excitability at a given time point. Irrespective of their generation mechanisms, most neuronal oscillations (see Figure 7), such as those in the delta (<4Hz), theta (4-8Hz), alpha (8–12Hz), beta (15–30Hz) and gamma (>30-80Hz) bands rely to some degree on the phasic inhibitory activity of GABAergic interneurons, either expressed at a local level or at a larger scale level.

By means of alternating ‘down-states’ of wide-spread neuronal silence, and ‘up-states’ of increased neuronal firing, neural oscillations carry information, subtend their processing across network sites, and also modulate plasticity[189]. From those basic features of brain activity, multiple questions about human cognition and physiology can be formulated. For example, one can explore how signals synchronize across regions at the sensor level (between scalp EEG electrodes) or source space (between brain sites on which EEG activity can be source-localised) and how they relate to human cognitive performance, or explore evoked, induced or elicited responses in the time domain, the frequency domain or both simultaneously. However, EEG signals are commonly artifacted by multiple sources of ‘noise’, defined as events not representing neuronal activity arising from muscle contractions, oculomotor activity (e.g., blinks, saccades, movements), peripheral collateral sensory inputs, bad contacts between electrodes and the scalp inducing signal drifts or artificial trends, cardio-vascular artifacts, or external electromagnetic sources of ‘noise’. All these sources of ‘noise’ often lead to data misinterpretations and they need to be carefully addressed with adequate experimental procedures and by pre-processing pipelines employing personalized combination of trial- or sensor-rejection algorithms, temporal filtering (aka. *frequency filtering*), independent component analyses and/or *spatial filtering*. In trial-to-trial experiments, signal to noise ratio is thought to be trial independent, thus, increasing the number of trials does not necessarily lead to improvements of the experiment readouts. Once data is cleaned, time-, frequency- or phase-domain analyses can be conducted.

Time-domain analyses are often used to examine event-related potentials (ERPs) in response to an external trigger event. ERPs are obtained by averaging the signal across trials at each time point (sum of the voltages at each time point over trials then divided by the number of trials)[190,191]**.** Each trial contains signal and noise; When trials are aligned and averaged through, signals that are constant on each trial tends to stay, whereas noise fluctuates randomly distributing their values around zero, hence cancelling out and leaving ERPs. In ERPs, only activity phase-locked to an external trigger stimulus will be represented, and every time point on this averaged signal can have different topographical representations across scalp sensors (sensor space) or their estimated brain sources (brain space). ERPs offer information about the amplitude of the evoked responses and their latency, allowing the objective exploration of the underlying levels of brain excitability. However, oscillatory components will not necessarily be time-locked to the triggering event hence not always represented in event-related fields and potentials. This is why, to pinpoint the contribution of oscillations to the research questions, *ad hoc* time frequency-analyses will be necessary (Figure 18).

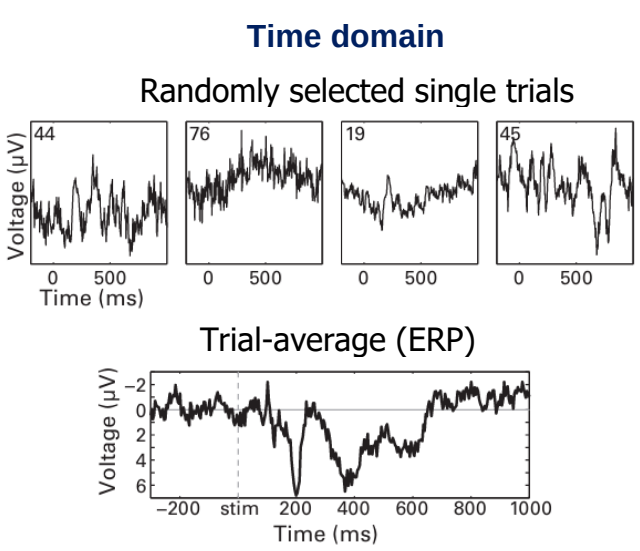


Figure 18. EEG time domain analyses: ERP. Cortical evoked activity that is time-locked to external events (e.g., visual stimuli, sensory inputs) can be recorded non-invasively on a trial-by-trial basis. Individual trials (upper panels) capture both evoked and induced neural activity. When a grand average across all trials is computed (bottom panel), the mean evoked activity can be quantified. This averaging process effectively reduces trial-to-trial noise and eliminates non-time-locked oscillatory components, isolating the event-related signal for analysis[188].

Analyses in the frequency domain explore the power (estimate of amplitude) of activity at a arbitrarily discretized continuum of oscillatory frequencies, referred to as bands (power spectrum) at a fixed time point or period (power x frequency) or across a time epoch (power x frequency x time)[192–194]**.** The analysis of power spectra require the isolation of neural activity at specific frequency bands related to neural processing: delta (1-4Hz), theta (4-8Hz), alpha (8–12Hz), beta (15–30Hz) or gamma bands (>30Hz). By employing Fourier transformation or complex waves (e.g., Morlet), EEG time-series can be translated ('transformed') in the frequency domain obtaining the so-called frequency domain transforms. To this end, progressive sinusoids or wavelets (or any kind of 'kernel filter') are used to estimate the convolution between the data time-series with such sinusoids or wavelets. For example, if a time-series has a particular cycle frequency (e.g., 3Hz oscillation) at a given time window (for example 1 second window with a signal oscillating for 3 complete cycles), a convolution is applied across phases compared to a 3Hz sinusoidal oscillation resulting in a positive phase-locking similarity index (aka '*dot product*'). However, another time-window of the same time-series signals with predominant activity at 40Hz, the comparison of phase locking

factors between this activity cycle and 3Hz sinusoids will be minimal, resulting in a low phase-locking similarity index. Overall, time-frequency transformation is the procedure by which we estimate the presence of oscillatory power depend on the oscillatory cycle and the maximum power relates to the relative original signal power over time. In signal processing, Fourier transformation is optimal if one assumes signal stationarity over time given that classical Fourier transform only keep a non-time resolved power spectra (i.e., frequency X power output), whereas, for non-stationary signals wavelet decompositions, Welch, short-time Fast-Fourier transformation or Hilbert transformation are mostly recommended, keeping time-varying power spectra (i.e., time X frequency X power output) (Figure 19).

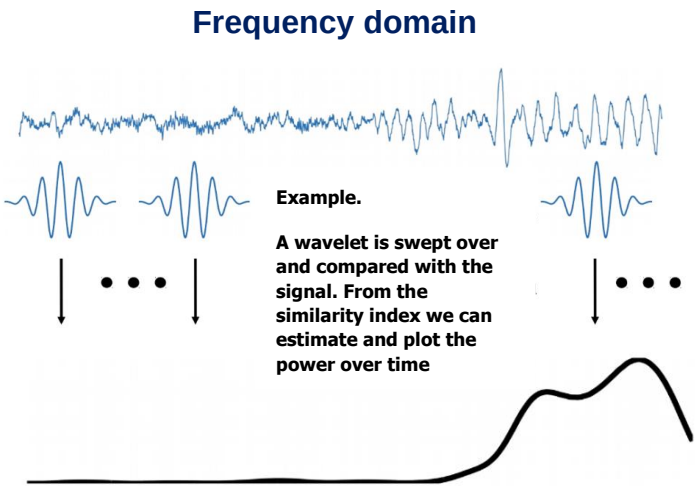


Figure 19. EEG frequency domain analyses. Frequency-domain analysis enables the quantification of both time-locked (evoked) and non-time-locked (induced) neural activity. By applying wavelet transforms to continuous, time-varying EEG signals, specific frequency components can be isolated and analyzed. A wavelet of a defined frequency is systematically swept over to the EEG time series, yielding a similarity index that reflects the degree of alignment between the wavelet frequency and the signal at a given time point. A high similarity index indicates prominent neuronal activity at the corresponding wavelet frequency. By utilizing wavelets across a range of frequencies, it is possible to examine the power of the entire spectrum while preserving temporal resolution, thus capturing the dynamic interplay of neural oscillations over time.

Finally, **oscillatory activity can be dissected into phases to explore local (or distant) synchronization features and determine the transition between excitation and inhibition as an indirect readout or proxy of the degree of excitability of the underlying neurons, part of an oscillator, at a given cycle time** [30]**.** For example, power and phase values can be extracted by employing a Hilbert transformation to the time-series data. Phase estimates rely on the frequency transformation of the signal, given that oscillations across frequency bands can exert different phases in the same time-window. Phases offer additional insights into signal characterization, for example enabling the examination of trial-to-trial synchronization. This can reveal the degree of oscillatory synchronization across trials in response to an external event (inter-trial phase coherence, ITPC) and various metrics related to the connectivity of neuronal populations. Such analyses allow to determine of these neuronal populations phase-lock over time and whether one source can drive the synchronization of another source (hence withhold '*effective connectivity*´). Beyond classical signal measurements, factors like background noise reflecting aperiodic activity (1/f component reflecting broadband activity decreasing with frequency, and the width of peaks in the power spectrum) and information theory metrics (such as entropy, signal predictability) can also offer valuable insights into biological systems[188,195], mainly reflecting background excitatory-inhibitory balance (Figure 20).

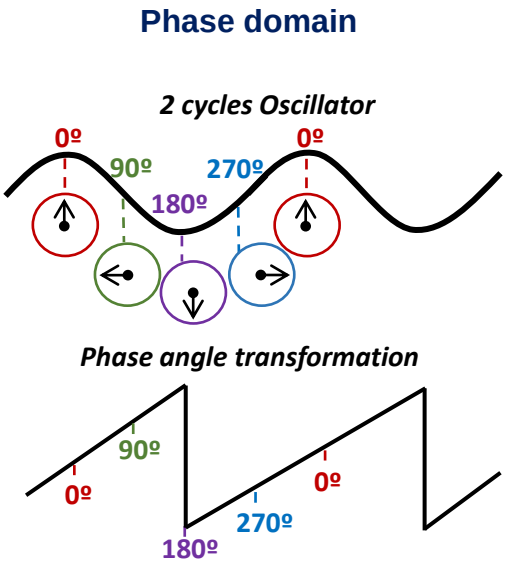


Figure 20. EEG Phase domain analyses. The phase of an EEG signal at any given moment represents the state of rapid excitability fluctuations within neural circuits. For instance, a 2 Hz oscillation (top panel) can be converted into its phase representation (bottom panel) to evaluate the degree of temporal synchronization between this oscillator and other distributed brain oscillators. By quantifying this alignment, phase-domain analysis provides crucial insights into how neural oscillators coordinate their activity over time, elucidating the mechanisms underlying inter-regional communication and synchronized neural processes.

In EEG recordings, frequency-, phase- or time-domain analyses are performed over a time-series recorded by a single electrode or a combination thereof in contiguous regions of the scalp surface. EEG systems are built with multiple simultaneous active electrodes (e.g., most commonly either 32, 64, 128 or up to 256 electrodes) placed on the scalp according to international reference systems such the 10/20 or the 10/10. In systems with multiple contacts (e.g., >32) it is possible to localize the cortical source of voltage fluctuations captured by the scalp data, a procedure known as *source localization* or *source reconstruction*.

As mentioned before, postsynaptic currents of neuronal assemblies generate local fluctuations in the net cortical charge evoking electric field potentials that can be recorded transcranially. However, most of the primary currents of evoked fields travel across the entire grey matter and cerebrospinal fluid, thanks to their high conductivity. Therefore, **the small proportion of currents reaching the skin that can be recorded with scalp electrodes are highly source unspecific.** EEG signals are a linear combination of multiple sources and can be explained with linear algebra methods. Several methods have been developed to find solutions to the ill-posed inverse problem that assumes the source estimation (i.e., estimate the strength or sensitivity of a source in a particular brain spot) of EEG/MEG recordings. The spatial resolution of linear estimates from EEG measurements is already limited by the fact that one cannot separate more sources than independent measurements, thus electrodes. Hence, the most straightforward way to separate activity from different source is to model activity only for sources of interest (e.g., location from where we assume activity to arise) and estimate how single dipole could best explain the data, a method referred to as *dipole fitting*[196–199]. Other methods like *linearly constrained beamformers*, considered and usually introduced as adaptive spatial filters, usually try to fit the activity of every individual source given a time-series data minimizing the source variance at a given location assuming that different parts of the brain cannot be simultaneously active[196–198,200,201]. A more general approach is taken by distributed source models, assuming these are equally likely to be active in the brain and attempt to find a source distribution that explains the data: this is the case of the minimum norm estimators[202].

However, the estimation of electrical time-series is still challenging due to crosstalk leakage between brain regions. Strategies to achieve better spatial resolution include:

the suppression of the cross-talk between a predefined region with respect to another ROI, the minimization of cross-talk from other locations anywhere else in the brain with respect to an ROI or the minimization of noise[203].

3.4. Concurrent TMS-EEG

The challenging use of concurrent transcranial magnetic stimulation patterns (either single pulses, bursts or long trains) with EEG is becoming increasingly popular in the NIBS field in order to probe the excitability of a cortical region/network, to functionally identify the presence and magnitude of time-resolved connectivity between a stimulated cortical region and other cortical target or targets, and to estimate the nature and direction of a instantaneous regional state. Electrical currents induced in the cortical gray matter by TMS pulses depolarize neuronal membranes opening voltage-sensitive ion channels and triggering action potentials. The so called **TMS-evoked and TMS-induced neuronal activity is then reflected in concurrent EEG recordings which uptake a linear projection of the distribution of postsynaptic currents** [146,150,204]. The first effort to combine TMS with electroencephalography (TMS EEG) was reported in 1989 by RQ Cracco and colleagues[205], reporting transcallosal responses after the TMS, accompanied with large EEG electromagnetic artifacts. A few years later in 1996, RJ Ilmoniemi sorted partially the electromagnetic EEG signal saturation and conducted the first successful concurrent TMS-EEG study demonstrating the feasibility of using this approach to estimate cortical excitability and characterize causal connectivity effects[155,206].

Since such pioneering studies, the combination of a causal approach such as TMS and a correlational method such as EEG both with excellent temporal resolution (see Figure 15 E) has opened new avenues for the neuroscientific community. The most distinguished advantage of this approach is the direct evocation and exploration of excitability and connectivity measures in the time- and the frequency-domain applicable to health and disease states. More specifically, a majority of concurrent TMS-EEG studies have focused in the generation and recording of Transcranial Evoked Potentials (TEPs), which are event related potential (ERP) evoked by single TMS pulses, and the entrainment of TMS oscillations, consisting in episodic periodic EEG activity evoked or induced directly by a TMS pulse or by a short train of pulses delivered by a given frequency. While any type of TMS coil and stimulation device could be used to evoke activity, *adhoc* TMS compatible amplifiers and scalp electrodes are required to handle artifacted TMS-EEG recordings from the same noise contamination as prototypical EEG plus the important addition of high amplitude 2 to 10 ms (depending on amplifier sampling rate) long electromagnetic stimulation artifacts generated by each TMS pulse. Overall if on a trial-by-trial basis, noise levels is superior to signal (signal-to-noise ratio), interpretation of EEG recordings can be compromised. TMS pulses induce different types of artifacts which have been extensively characterized by the TMS-EEG community[207,208]. They need to be minimized during experimental settings or filtered out by mean of special post-processing data procedures and can be either *time-locked* or *non-time-locked* to the onset of a TMS pulse and also divided in *physiological* or *non-physiological*. Direct electromagnetic discharges, recharging artifacts, electrode polarization decay artifacts or electrode mechanical vibration are common *non-physiological* artifacts generated by TMS. Physiological artifacts encompass TMS evoked eye blinks or eye movements, cranial or face muscle contractions, auditory

evoked effects induced by the TMS clicking noise or peripherally-induced responses such tactile/mechanic stimulation of skin receptors, cranial nerves or bone vibration conduction. Some of these artifacts can be minimized during experimental settings, such as exposure to white noise via earplugs to minimize auditory artifacts, or the addition of a foam pad between the TMS coil and the scalp to partially supress somatosensory stimulation. Additionally, very low skin electrode impedances (<10-5 kOhm with passive electrodes, <25-35 kOhm with active electrodes) help to minimize line noise, signal decays and polarization artifacts. Electromagnetic artifacts can be minimized with preprocessing steps in data analyses, however TMS-EEG data represent by essence network activity mixed with peripheral multisensory responses.

Preprocessing in TMS-EEG is not a straightforward procedure because different experimental technical settings (e.g., a combination of EEG amplifiers, EEG electrodes, TMS coils and their scalp sites and brain targets, TMS electronics, etc.) can result in different artifact types. Accordingly, the cost/benefit ratio of a given approach for signal-processing depend on the validity of the underlying assumptions. Nevertheless, some steps are similar across laboratories and should be performed independently of the experimental settings. Commonly used preprocessing steps encompass the data *epoching* around the TMS pulse (TMS pulse = 0ms), removing or interpolating bad channels or trials, removing and interpolating TMS window artifacts (artifact length depending on sampling rate), re-referencing the data (mean reference/average reference), baseline correction (for instance -500 to -10ms), minimize or supress when possible large amplitude artifacts like ocular movements, current decay, electrode polarization or TMS induced muscle activity, by fitting a model with a control condition and subtracting it from the signal using: blind source separation algorithms such PCA or ICA, source based artifact rejection technics like the signal space projection with source informed reconstruction SSP-SIR or source-estimate-utilizing noise-discarding algorithm SOUND and temporally filtering with low-pass, band-pass, and band-stop “notch” filters to remove low-frequency drifts and residual line noise, with a final down sampling of the data to 500 or 1000 Hz[209–215] (Figure 21).

Additionally, to ensure that the data cleaning procedure did not alter EEG signals outside of the brief period corresponding to TMS artifact duration and to attest that artifact removal and cleaning procedures could not explain the observed changes in evoked TEPs or entrained oscillations, a series of control analyses can be conducted as ‘sanity checks’. For example, TMS artifacts can be artificially added to series of well-characterized components EEG signals obtained at the resting state from the same participant during experimental sessions (hence with known evoked and oscillatory components in terms of power or ITC) then submitted to the same cleaning procedure as the original TMS-EEG data to assess first how accurately one could extract similar evoked or oscillatory activity patterns as the one originally present in the trace, to finally compare outcomes in originally artifacted trials vs. artificially artifacted trials[216].

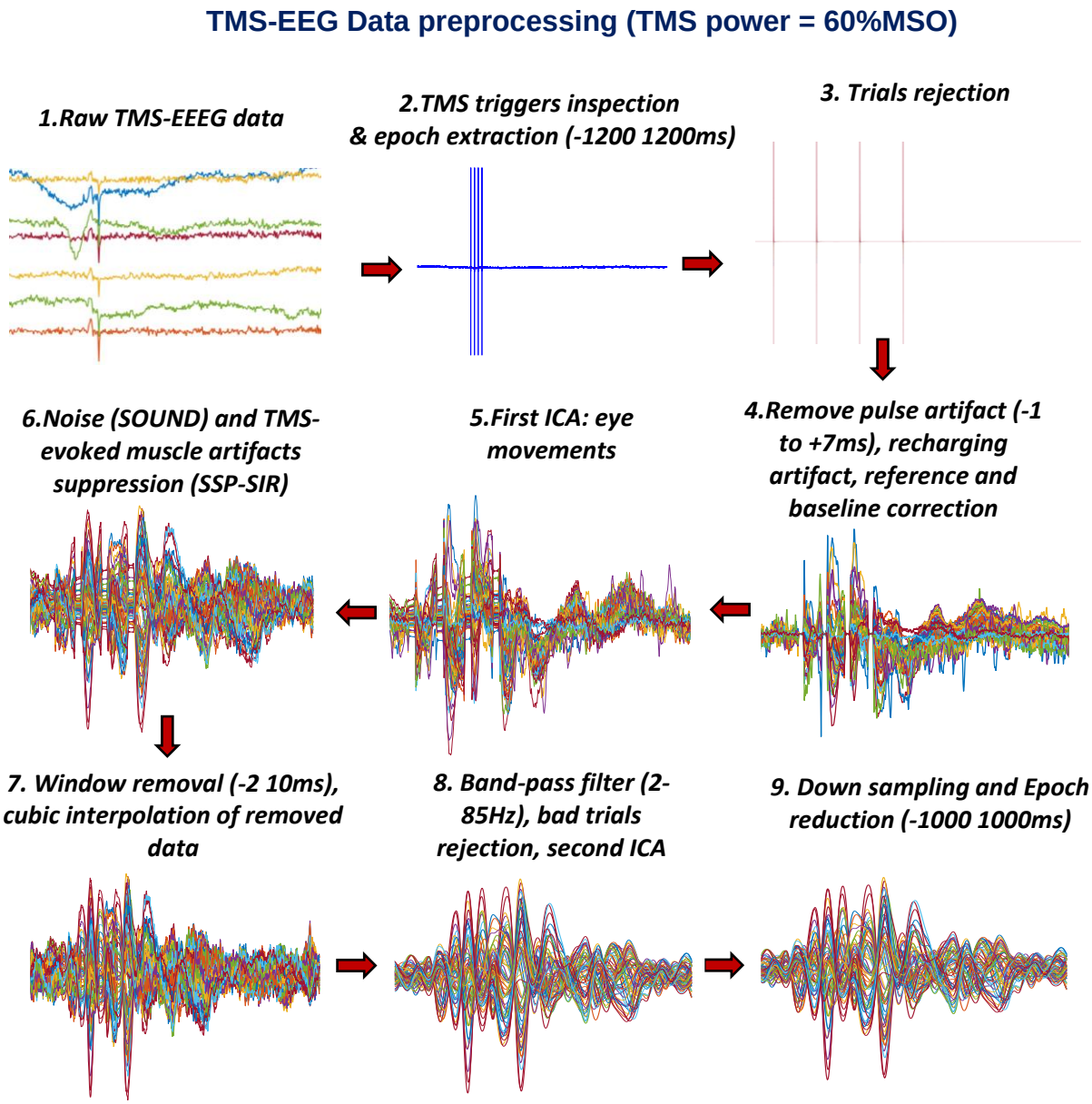


Figure 21. TMS-EEG preprocessing example. Example of data preprocessing and cleaning pipeline applied to a TMS-EEG data acquired during a 4-pulse rhythmic (20Hz) burst stimulation protocol, where TMS is delivered at a fixed intensity of 60% of the maximum stimulator output (MSO). The steps for cleaning artefacts in TMS-EEG data vary depending on experimental and equipment setups, but some general approaches can be applied across scenarios. Initially, raw data is epoched around the TMS pulse or bursts, and electromagnetic artefacts as well as recharging artefacts are removed and interpolated. Following, baseline correction is applied, and independent component analysis (ICA) is used to remove large artefacts such as eye movements or muscular contractions. Additional algorithms, such as SOUND or SSP-SIR, can then be used to address line noise and residual muscular artefacts. Finally, band-pass and notch filters are applied to eliminate low-frequency trends and high-frequency non-physiological activity (see[214] for more details regarding the impact of different artifact removal approaches). All signals and visualizations were processed and generated using the TESA, EEGLAB, and FieldTrip toolbox.

Outcome measures to assess the impact of TMS can encompass any EEG analyses in time-, frequency- or phase-domains. Nevertheless, **the most commonly explored features in TMS-EEG datasets have been: Local TMS evoked potentials (TEPs), Event Related Spectral Perturbations (ERSP) and Intertrial Phase Coherence (ITPC) measures**, employed respectively to study evoked activity, brain oscillations and effective connectivity. TEPs are brain potentials time-locked to the TMS pulse onset obtained by averaging single-trial signals across trials. The initial TMS evoked response (~1-40ms) is produced by the activation of neurons in the targeted area, and activity that is followed by the activation of interconnected or multisensory activated regions. The maximal amplitude is recorded by EEG electrodes close to the stimulation site. TEP peaks and time courses depend on the stimulated area, coil orientation and state of activity in the underlying cortex (determined by task-engagement, resting state activity, or oscillatory phase).

Likewise, TMS effects on brain activity can be investigated in the frequency domain. When a cortical site is stimulated by a TMS pulse of sufficient intensity, this resets the phase of the underlying oscillators as measured by EEG and these tend to oscillate in normal conditions at their natural intrinsic frequency [37,216–219]. Accordingly, by measuring the impact of a single TMS perturbation pulse on EEG signals, concurrent TMS-EEG recordings can be used to investigate brain rhythms characterized as ERSP. In rhythmic repetitive TMS conditions, scalp stimulation patterns can phase-reset the underlying cortical oscillators and generate signs of neural entrainment by amplifying over the course of a TMS train oscillatory power. TMS-*elicited oscillations* (time locked + non time-locked oscillations) can be further subtracted from *evoked oscillations* (time locked oscillations) (by averaging trials before frequency transformation) to obtain *induced oscillations* (non time locked).

Finally, common TMS-EEG phase domain analyses encompass the study of phase consistency across trials, also referred to as *Inter-trial Phase-locking factor* or *Coherence* (ITPC)[220]. ITPC values range from 0 to 1, where values close to 0 reflect high variability of phase angles across trials, and values close to 1 high consistency. ITPC is not a connectivity measure, as it does not relate the phase in a EEG channel to that of another EEG channel. ITPC is mostly related to the phase locking capabilities of a TMS pulse to synchronize underlying oscillators (Figure 22).

While the most common and well-established analyses in the TMS-EEG field include TEPs for assessing cortical reactivity and excitability, ERSP for exploring oscillatory features, and ITPC for investigating phase resetting and local synchronization phenomena, signals can also be analyzed using alternative approaches. These methods can focus on periodic components, such as time, frequency, and phase, or examine non-periodic components. For instance, phase synchronization metrics (e.g., phase-locking value or imaginary coherence) applied across sensors after the TMS pulse can provide insights into potential long-distance synchronization events between brain regions. Similarly, global or local mean field power can offer valuable information about the predominant spectral frequency over time, either in the stimulated cortical area or across broader brain regions. Moreover, exploring aperiodic components of the signal (irregular activity without a characteristic frequency, often referred to as 1/f-like, fractal, or scale-free activity) may shed light on the underlying excitatory/inhibitory balance in neural dynamics. However, further validation and standardization of these advanced analytical methods are essential to solidify their use in TMS-EEG datasets and to prevent potential misinterpretations.

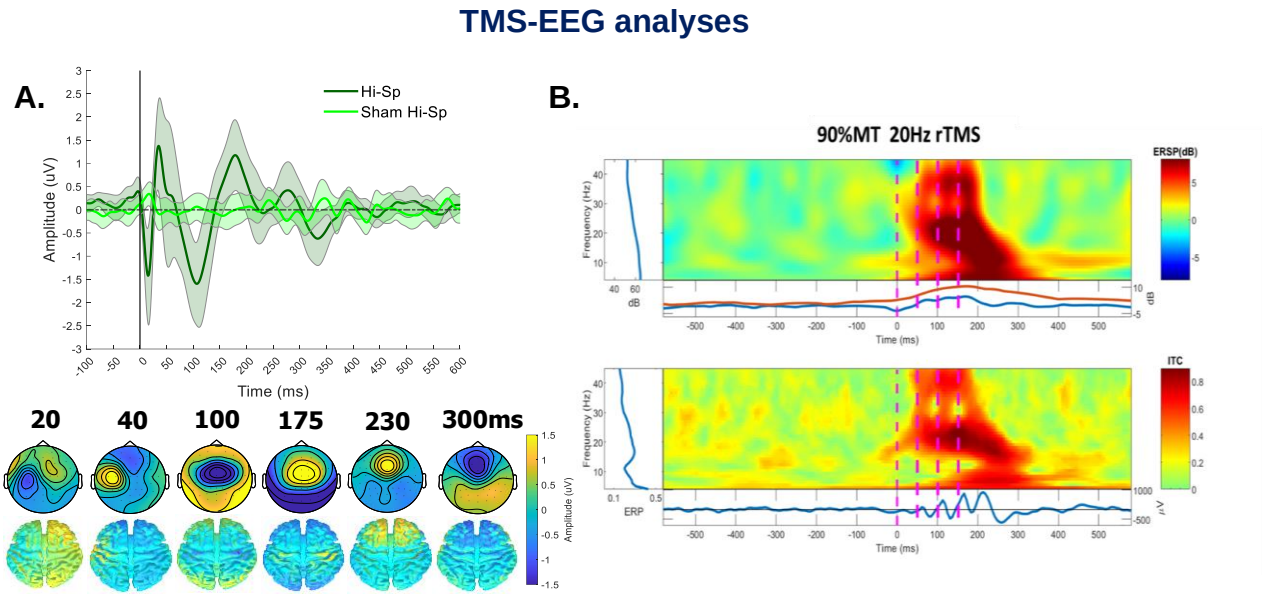


Figure 22. TMS-EEG example analyses. A. Example illustrating single-pulse TMS-evoked potentials (TEPs) in a experiment where TMS was applied to the left motor cortex. Sensor-level and source-level topographies highlight classical components associated with locally evoked activity in the early stages following the TMS pulse (0 to 40 ms). These are subsequently followed by topographies reflecting multisensory-evoked activity, including auditory and peripheral muscular or sensory events, which exhibit a fronto-central distribution. **B.** Example of entrained TMS evoked potentials, spectral perturbation and phase trial locking factors illustrated in a rhythmic 20Hz TMS-EEG (4TMS pulses) dataset, delivered to the left motor cortex at 90% of the individual resting motor threshold. Beyond single pulse evoked activity, repetitive stimulation demonstrates clear entrainment effects after 20Hz rhythmic TMS across the analyses of local EEG signatures (sensors under the TMS coil after data preprocessing). TMS can entrain endogenous natural oscillations if delivered at sub-action potential threshold intensities, or entrain/impose external frequency-specific rhythms when delivered at supra-action potential threshold intensities. The upper figure correspond to the time-frequency Morlet wavelet transformation of elicited oscillations (ERSP). Inferior figure corresponds to the inter-trial phase coherence (ITPC). Left adjacent panel depicts mean power spectrum. Bottom adjacent panels depict mean spectral envelope (for ERSP plot) and the TEP = ERP time-series (for ITC plot). Data extracted from TMS-EEG experiments included in this thesis.

3.5. Statistical analyses and cluster based comparisons

Statistical comparisons between low-dimensional datasets can be implemented with classical statistical approaches after testing for data normality (e.g., Shapiro-Wilk test) and applying either parametric testing when a normal distribution can be assumed (e.g., T-tests, ANOVAs, ANCOVAs) or non-parametric approaches when normality criteria are not fulfilled (e.g., Mann-Whitney, Wilcoxon, Kruskal-Wallis) followed by post-hoc multiple comparisons (e.g., Bonferroni, Tukey, Dunn's test). Bayesian statistics or regressions can also be employed. However, EEG data is characterized by a high dimensionality distributed along sensor, time and the frequency variable space. For example, pre-processed data from our experiments (from *Experimental section 2*) is composed by 63 electrodes, at 1Khz sampling rate (1000 data points/second), totalling in a single trial lasting 1 second, 63000 data points. Consequently, the statistical comparison of EEG topographies, time courses or time-frequency maps entails the Multiple Comparison Problem (MCP). **When conducting multiple statistical**

comparisons (such as for each electrode or time point), the likelihood of obtaining a false positive in a statistical test (family-wise error rate) is high and needs to be corrected by considering more than just individual p-values[221,222].

Although several methods are available to correct p-values depending on the normal distribution of the data (Tukey, Bonferroni, the False Discovery Rate), the most often employed method to evaluate behavioural, neuroimaging or neurophysiological datasets is the Permutation Test. This approach serves to examine whether the null hypothesis is true; hence if samples in a let's say 'A' and 'B' conditions or groups follow the same probability of distribution. If the null hypothesis is retained, the permuted data can be considered identical as the real biological data, whereas if the null hypothesis is false, it signifies that the permutated data will be different from the real biological data. Nevertheless, for most EEG datasets the number of potential permutations is far too high and calculating an exact permutation test is computationally demanding.

Taking advantage that in EEG data datapoints cannot be considered totally independent and that neighbouring points are highly correlated, we can employ a clustering followed by permutation tests with the *Monte-Carlo sampling procedure* to observe trends in the data and reduce the above-mentioned computational costs. Therefore, the MCP can be solved in permutation tests by comparing test statistics to a null distribution obtained from a statistical representation of the complete data space (i.e., cluster). On such scenario, the *Monte-Carlo sampling procedure* is used to draw a subset of all possible permutations to estimate the null distribution exclusively in this representative subset. The higher the number of permutations drawn, the more accurate the p-value estimated with Monte-Carlo sampling will be. It should be noted that any

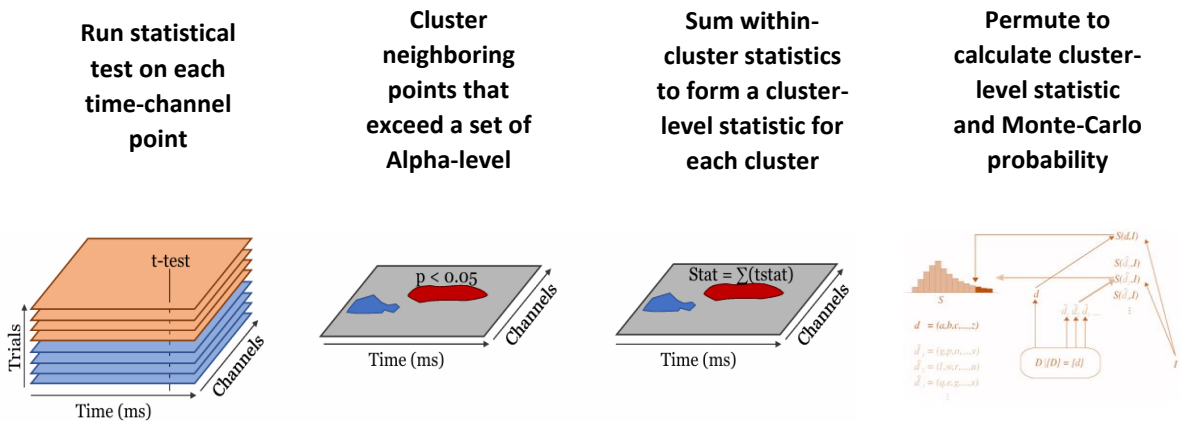


Figure 23. Cluster-based statistics. Diagram of EEG cluster-based permutation statistics used to determine the statistical significance of high-dimensional time-series EEG data. To assess whether two time-series significantly differ across time, t-tests are initially performed on each time-channel data point. Next, neighboring points that exceed a predefined alpha threshold (e.g., 0.05) are grouped into clusters, and a cluster-level statistic is calculated by summing the values of all points within each cluster that meet the significance criteria. Following, The multiple comparisons problem is addressed using the Monte Carlo method. This involves permuting the dataset by randomly shuffling trials between conditions a large number of times (e.g., 5000 iterations). For each permutation, the cluster-level statistics are calculated, and a Monte Carlo distribution is generated from the largest cluster statistics of each permuted dataset. Finally, the proportion of random permutations that produce a cluster-level statistic greater than the original data determines the Monte Carlo significance probability (the p-value).

physiological effects recorded on EEG datasets are unlikely to be localized at a single electrode in the sensor space, instead this effect will be spread over adjacent contacts.

To this regard, source-based reconstruction of time-series data prior to statistical testing can help to disentangle the source origin of the statistical difference, however, at the expense of increasing the computational cost, each source of the forward model contains an individual reconstruction of the time-series data (e.g., 63 electrodes time series data is transformed into 2000 source time-series data).

In the analyses included in parts of this dissertation (see TMS-EEG studies in *Experimental section 2*), we performed data cluster-based non-parametric permutation tests with *Monte Carlo sampling*. First, each pair of data points between two conditions were compared with two-tailed paired Student's t-tests. Then, adjacent data points that exceeded the cluster significance threshold (alpha=0.05) were clustered together and the sum of t-statistics of each point in the cluster was assigned as the statistic of the cluster.

Then, to explore if clusters were likely to arise from chance alone (family wise error), the cluster statistics were compared by shuffling the trials between conditions a high number of times (e.g., 10000 times), calculating the cluster-level statistic for each newly generated dataset, building a Monte-Carlo distribution of each dataset's largest cluster (the test statistic) and finally calculating the proportion of random permutations that resulted in a larger statistic than the original, providing the Monte-Carlo significance probability (final p-value) (Figure 23). Any clusters exceeding the significance threshold (alpha=0.05) in the permutation test was considered to show a significant difference between conditions.

3.6. Electric field modelling

TMS and tDCS are technologies allowing the injection of electrical current in the brain and the modulation of neural activity. The spatial focus, extent and distribution of the so called current electrical field (E-field) determines the region directly affected by the external forces and the evoked effects observed through concurrent NIBS-EEG (or other neuroimaging technics). However, inter-individual differences in cortical folding patterns or inhomogeneities of conductivity throughout tissue layers from the scalp to the brain surface impacts electric field dispersion and penetration properties. **Accurate estimation and prediction of how electrical fields project into participants heads may improve our understanding of the mesoscopic and microscopic effects of stimulation, improving target engagement strategies**[223–232]**.**

In TMS scenarios, electric fields can be mathematically described as the sum of two terms: the *primary electrical field* and the *secondary electrical field*. Since TMS coils are typically flat and placed tangential to the scalp during stimulation, the resulting E-field in the brain is also tangential to the scalp. Generally, the distribution of the electric field induced by the TMS in a cortical surface will depend on the shape of the TMS coil, the location and orientation of the coil, the capacitator and coil combination as well as the *conductivity* (aka *permittivity*) or *resistance* properties of each layer of tissue the current needs to go through to reach the cortical grey matter.

The *primary electrical field* is a direct effect of the coil's rapidly changing current. The amplitude or density of a primary field depend on how quickly the electric current flows through the coil (speed of variance or rate of charge: *dI/dt*). Rapid changes in current (high *dI/dt*) result in stronger magnetic fields and thus higher induced electric fields in brain tissue. Normally, the values of *dI/dt* are reported for convenience, in units of A/µs or A/s. Charge rate depends on the TMS coil and machine combination and needs to be estimated *a priory* in every machine for precise E-fields simulation. Typically, TMS intensity is expressed in maximum stimulator output (MSO) units, which refer to the relative to the maximum stimulation intensity being produced by a TMS device (and its attached TMS coil). This parameter, MSO, is highly ambiguous as it varies across rTMS machines and coils. Nevertheless, the linear relationship between MSO and charge rate (A/µs) within coil and machines simplify MSO interpretations across intensities (e.g., 100%MSO of a Magstim200 and figure-of-eight-coil = 114,7 A/µs / 50%MSO of a Magstim200 and figure-of-8-coil = 57,35 A/µs). Given that the polarization of neuronal structures is expressed according to their membrane voltage potentials (V/m or mV/mm), NIBS E-field simulations are express similarly. Electric fields (E) are the force per unit charge, while volts per meter (V/m) are units of measurement for an electric potential (V) describing the potential voltage difference ($E = -\nabla V$) per distance. It is important to remember that most TMS machines use biphasic pulses, therefore TMS induce a phasic modulation and phasic time-varying dynamic E-field (intensity varies over the TMS pulse)[155,224,228,233]. Mesoscopic E-field modelling represents the peak intensity of the field whereas over the pulse, the intensity impact varies.

The primary electric field can be described according to Faraday's law:

$$E_1(r,t) = -\frac{\partial A(r,t)}{\partial t}$$

where E is the primary induced E-field and A is the magnetic vector potential at position r and time t. The vector potential A is given according to the Biot-Savart law and can be formalized as:

$$A(r,t) = \frac{\mu_0}{4\pi} I(t) X \oint_C \frac{dI(r')x(r-r')}{|r-r'|^3}$$

where A is the magnetic vector potential, μ_0 is the permeability of free space along the coil windings C with I current density of the TMS.

As mentioned, tissue conductivity has as essential effect on the E-field distribution whereas charge accumulation at conductivity boundaries causes a *secondary E-field*, which may in some cases, dominate the primary one. Therefore, the resulting magnetic field is the sum of a primary and secondary one. Thus, equations to estimate magnetic fields in the brain can be formalized as follows:

$$E = E_1 + E_2 = -\frac{\partial A}{\partial t} - \nabla \Phi$$

with A representing the magnetic vector potential of the TMS coil, and Φ the electric scalar potential that results from surface charges accumulating at discontinuities of the electrical conductivity distribution. The vector potential of a coil can be determined by subdividing the coil area into many small magnetic dipoles defined as:

$$m = I(t) D e_n$$

with *I(t)* representing the current density in the enclosing coil wires, *D* depicting the area of the part of the surface modelled by the dipole and e_n the surface normal. The vector potential of a magnetic dipole is given by:

$$A_{dipole}(r,t) = \frac{\mu_0}{4\pi}\, m\, X\, \frac{r - r'}{|r - r'|^3}$$

with *r* indicating the space positions at which the field is evaluated and r′ indicating the dipole at time *t*. Finally, the total vector potential induced by the TMS coil at position *r* (e.g., a source position in a brain head model) is then obtained as the summed field for all dipoles, and can be separated into two functions describing time dependence and spatial distribution:

$$A_{coil}(r,t) = \sum_i A_{dipole,i}\,(r,t) = f(t) \sum_i A^S_{dipole,i}\,(r)$$

The secondary fields (E_2) are computed according to Maxwell's equation $\nabla E_2 = \frac{p(r,t)}{\varepsilon 0}$, where p is the charge density and $\varepsilon 0$ is the permittivity of free space, which can be expressed as $E_2 = -\nabla \Phi$ representing the spatial gradient which obey Laplace's equation $\nabla^2 \Phi = 0$. For human brain stimulation, E-field modelling is typically conducted by employing a quasi-static approach (QSA), which simplifies the modelling equations to support tractable analysis, enhancing understanding and computational efficiency. The application of QSA is based on four assumptions: no wave propagation or self-induction in tissue, linear tissue properties, purely resistive tissue, and non-dispersive tissue. Consequently, each tissue is assigned with a fixed conductivity, and simplified equations are solved for the spatial distribution of the field separated from the field's temporal waveform. Several methods can then be used to solve the mathematical equations for the electric field inside the brain. For most of these methods, the brain is first discretized into small elements. Next, a solution such as the finite element method (FEM), finite difference method (FDM), the boundary element method (BEM) or the impedance method can be used. For the purposes of the present PhD dissertation, FEM methods were used. Finite element models are numerical solutions used to mesh a computational 3D surface into a finite number of smaller non-overlapping elements (commonly with a triangular or tetrahedral geometry). Each element is then assigned with specific properties like conductivity or density to study its behaviour under experimental conditions. Those properties can be isotropic (some conductivity in all directions of the element) or anisotropic (conductivity varies according to the current flow). Typically, to solve the E equations, A is first calculated at each node of a volume (head mesh) which is then used as input for the FEM solver to calculate Φ and finally E.

The modelling of tDCS currents (or any tCS variant such as tACS or tRNS) poses similar problem. The electric fields generated are given by $E = -\nabla \Phi$, and the calculation of the vector potential is very similar to the TMS described under the quasi-static regime. The main difference is that no volume source term occurs (magnetic vector potentials in every mesh node), but a *Dirichlet boundary condition* (as tDCS assumes pre-specified constant potentials) for the anode and cathode nodes is applied. By doing so, the electric potential Φ is set to predefined values at the skin surface corresponding to the anodal or cathodal electrodes. Electric fields are governed by the Laplace equation, meaning that the total electric fields $E(p)$ created by the current injected through multiple electrodes (e.g., n=2) refer to the sum of the fields $E_i(p)$ caused by the flow between an active electrode and the reference electrode:

$$E(p) = \sum_{i=1}^{n-1} E_i(p)$$

where p denotes a spatial position on the mesh, and E the fields created by a current flow between the i electrode and the reference. In the *Dirichlet condition*, models should account for the presence of electrode materials, including any conductive rubber, saline-soaked sponges, or electrolyte conductive gel. E-field simulations can be computed in spherical models to explore TMS coils capabilities. In real-world scenarios, structural magnetic resonance images (MRI) are used to segment high-resolution meshes[228,229,234,235]. In neuroimaging, several packages have been developed to segment the different cortical and scalp layers in human realistic MRIs and reconstruct the cortical hemispheres (SPM, FSL, free-surfer, CAT, etc). The resulting triangle surfaces represent the basis for building realistic head models and project E-fields (Figure 24). Current software allows to reconstruct multiple layers such white matter, grey matter, skull, or cerebrospinal fluid. In simulation environments, arbitrary conductivity values arising from realistic measurements and released in public literature can be assigned to every layer. For example, isotropic conductivity for the different brain

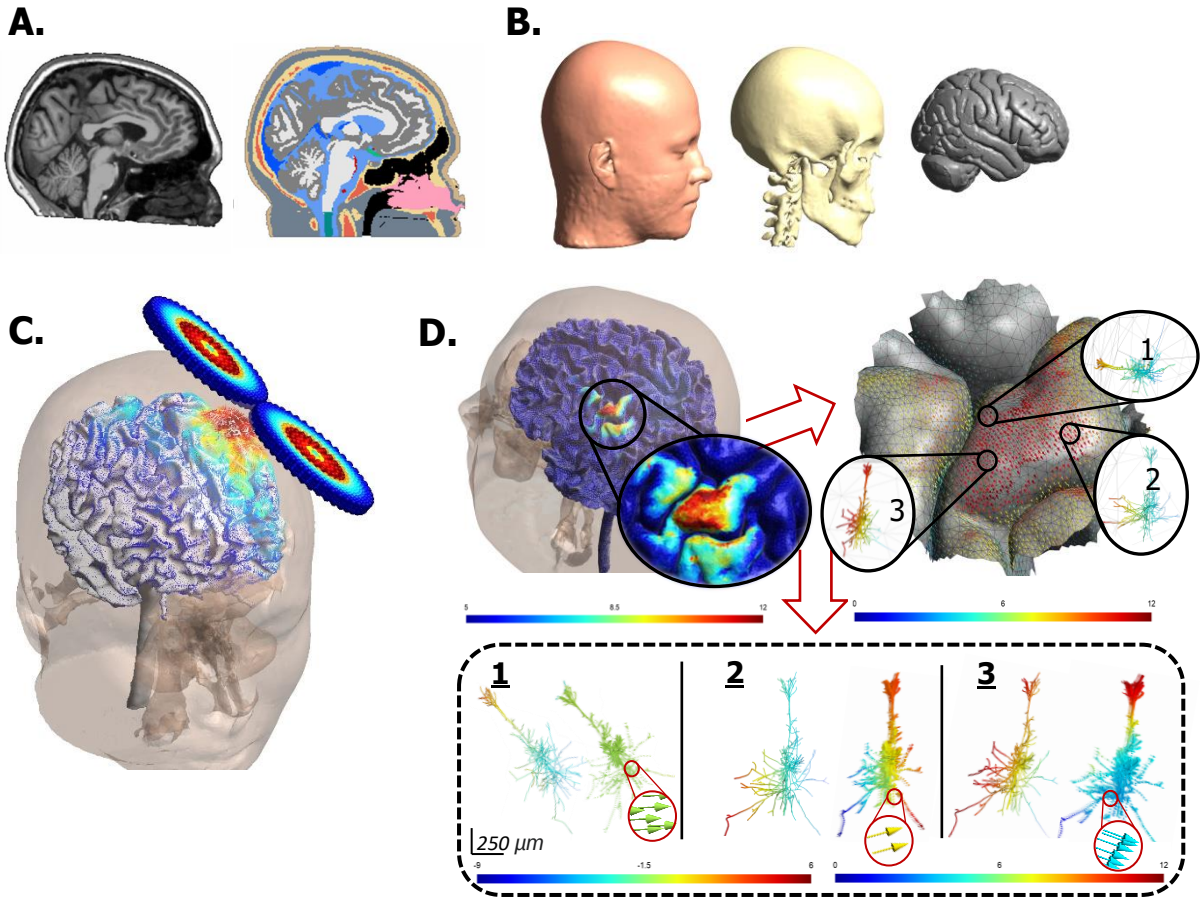


Figure 24. E-field modelling in TMS and tDCS. Computational numerical modelling is used to estimate the peak and distribution of the electrical fields (E fields) induced by NIBS technologies on brain anatomy. **A.** Example of a subject segmented structural MRI. **B.** Head mesh Finite Element Model (FEM) reconstruction from a single subject. Brain layers such skin, skull or grey matter (GM) are individually reconstructed and E-field projected. **C.** Illustration of a macroscopic E-field TMS simulation. **D.** Example of a TMS simulation across macroscopic and microscopic layers with embedded L5 pyramidal neurons with different spatial orientations.

layers have been typically assigned as follows: white matter (WM), 0.126 S/m, grey matter (GM), 0.275 S/m, cerebrospinal fluid (CSF), 1.654 S/m, bone, 0.01 S/m, scalp, 0.16 S/m, eyes, 0.5 S/m, compact bone (CB), 0.008 S/m, spongy bone (SB), 0.025 S/m, blood vessels, 0.6 S/m, and muscles, 0.46 S/m[236,237]. Nevertheless, conductivities in human are uncertain and non-stationary, and novel methods based on EEG signals conductivity quantifications, tensors derived quantification or employing Generalized Polynomial Chaos Expansion (gPC) for evaluating the effect of uncertainty in conductivity values are used[232]. E-fields are in most scenarios projected into the mesoscopic layer of the cortex; however, microscopic single cell modelling has emerged during the last decade as a reference method to predict dynamic polarization and calcium concentrations in single cells[181,238,239].

Nowadays, an extensive library of morphologically realistic computationally reconstructed cells exists and is used to embed cells in the cortical layers of macroscopic simulations. Briefly, based on the neuronal morphology, discretized numerical models of the neuron (generating independent compartments across the neuros) are segmented to extract time-series polarization and calcium concentrations from every compartment. To couple macroscopic E-fields with neurons and extract extracellular potentials or equipotentials from every compartment, cable theory computations provide the framework. Despite the flexibility of the methods and the potential for informing TMS in *pre-hoc* (e.g., planning prior to experiment or intervention to customize treatment) or *post-hoc* analyses (e.g., assessing correlations between the final behavioural/clinical impact and the estimated E-field calculation), microscopic modelling assumes in many occasions the stationarity of the neural system. Nonetheless, brain state changes dynamically and the predictions established by biophysical models may fail in biological realistic conditions.

3.7. Behavioural and clinical assessments of voluntary motion

A diverse range of experimental paradigms and clinical evaluations has been employed to examine motor abilities and adaptation as indicators of motor learning along the third experimental section of this thesis. **In clinical contexts, motor and cognitive functions must be evaluated according to the symptomatology of individual diseases, with qualitative and quantitative clinical tests and evaluation scales tailored to specific disorders to enhance the sensitivity and specificity of the assessments.** Stroke can lead to a wide range of disorders depending on the localization and extent of the cortical damage. Our research has focused on investigating the physiology of disrupted motor systems and functions in patients affected by a middle cerebral artery (MCA) stroke. Therefore clinical assessments were used to evaluate and quantify impairment of motor functions associated with neurological deficits causing direct damage to descending corticospinal tracts and basal ganglia-thalamic control systems resulting in symptoms such as paralysis (hemiplegia/paresia or paralysis), axial instability, ataxias, chorea, dystonia, tremors, myoclonus, bradykinesia, rigidity, dysfunction of reflex activity or spasticity by upper motoneuron syndrome and a decreases in descending inhibitory control. To evaluate the multidimensionality of symptoms arising from MCA strokes, clinical evaluations carried out in *Experimental section 3* of this dissertation includes the Fugl-Meyer Assessment (FMA)[240], the

National Institutes of Health Stroke Scale (NIHSS)[241,242], the Montreal Cognitive Assessment (MoCA)[243] and the AX continuous performance task (AX-CPT)[244].

The FMA is a widely employed considered a reliable quantitative measure for motor hemiplegic dysfunction used to assess motor impairment. It includes subsections evaluating the articular range and strength of multiple movements, coordination and spinal reflexes. For intra-subject pre-post-intervention assessments, an improvement greater than 6 points is usually defined as clinically significant. The NIHSS is a quantitative scale of post-stroke neurologic deficits widely used to characterize baseline post-stroke impairments and the prognosis of general health state. It explores consciousness, visual field, language function, hemineglect, hemiplegia, tremors and bradykinesia, and impairments of sensory function. Additionally, we included complementary measurements assessing cognitive states contributing to voluntary motion. The Montreal Cognitive Assessment (MoCA) is a quantitative screening tool used to explore stroke-related cognitive deficits including visuo-spatial and executive function, working memory, short-term memory, sustained attention, abstraction, language, and orientation in time and space. Finally, the AX-CPT is a computer-based paradigm designed to explore sustained attention and cognitive control subtended by prefrontal systems.

Alternatively, cognitive control of voluntary motion (e.g., learning and adaptation) can be quantified by assessing adaptation degree to external perturbations or learning degree of new movements or sequences[245]**.** While clinical scale-based evaluations provide information about a subject's functional motor abilities, experimental tasks offer insight into our capacity to (re)learn or (re)adapt new movements, a function for which cognitive control of motion is paramount. This can be evaluated through experimental computer-based paradigms exploring either *sensorimotor adaptation* or *sequential learning. Sensorimotor tasks* are classically associated with the acquisition of novel kinematics, such as goal directed movement speed and limb coordination, and dynamics, including muscle force and joint coordination. In contrast, sequence learning paradigms are associated with the acquisition of new motor sequences with lesser execution demands, as they rely on visual stimuli and require pressing a keyboard key via primarily the activation of hand muscles operating a given finger. Thus, while *sensorimotor tasks* and *sequential learning tasks* are both useful paradigms to examine the neural substrates subtending motor learning, their task demands may differ considerably. To this regard, metanalyses of fMRI studies have shown that both families of tasks, activate the central core of the motor system hence the dorsal premotor cortex (dPMC), the supplementary motor cortex bilaterally (SMC: comprising both the SMA-proper and pre-SMA), the primary motor cortex also bilaterally (M1), the left primary somatosensory cortex (S1), the left superior parietal lobule (SPL), the left thalamus, the putamen bilaterally, the prefrontal and the parietal cortices and multiple clusters in the cerebellum. Nevertheless, sensorimotor tasks have also shown a consistent activation of the striatum and right and left M1 as well as the both putamen nuclei and multiple areas of the cerebellum, thus are also considered excellent paradigms to probe cerebellar contributions to learning adaptation.

Since motor dexterity is usually significantly following and MCA, sequential learning tasks such as Serial Reaction Time Tasks (SRTT) are extremely hard to perform in mild-to-severe patients with hemiplegia or upper limb paresia/paralisis. For this reason, in the current dissertation, we explored learning through a classical Visuomotor Adaptation task[246–249] in which participants held a joystick with their impaired paretic upper limb hand. The joystick position was represented as a 1-cm diameter red dot (1º of angle) of

in a computer screen. Participants were asked to complete a series of consecutive trials to bring the red dot cursor from the centre of the screen towards a randomly allocated target displayed as a circle in several eccentric positions in the periphery.

Participants had limited time (10 seconds) to accomplish this task. After the time limit, or a successful placement of the dot inside the target, participants had to re-place the dot in the starting position to complete the trial. During the inter-trial interval (1.5 secs), a full grey screen with a central stimulus (“+”) was displayed to keep participants alert and ready for the subsequent trial. The task was divided into 3 consecutive blocks: *baseline*, *adaptation*, and *washout*. In stroke hemiplegic patients, since the task can be difficult, only two blocks were performed: The first block (*familiarization*) consisted of 32 trials and was followed by a second block *(motor adaptation*) including 64 trials. During the motor adaptation block, a “rotation-field-like” perturbation was implemented by introducing a constant angular rotation between the trajectory of the red dot and the movement of the joystick, deviating the trajectory of the former and forcing participants to compensate for such shifts in order to reach the target, hence engaging visuo-motor adaptation (learning) skills (see *Experimental section 3* for further details).

Once the task is completed, multiple analyses can be carried out on the learning or trial-by-trial readaptation curve to explore learning. For example, one can explore the degree of adaptation (difference between baseline and adaptation curves), the speed of learning (differences of area under the curve of the learning trials), electrophysiological EEG correlates of motor execution, preparation and performance, or in case feedback or reward is provided after each trial, to assess the role of reward and punishment in learning. Importantly, this paradigm allows to quantify cognitive control of voluntary motion and the implication of brain regions (when concurrently probed with NIBS) in learning scenarios (*see Manuscript 6* for extended methodological details).

4. RESULTS

4.1. Experimental section 1 – Personalized procedures for causal brain-behavioural exploration

This dissertation is formalized by a collection of articles. For a fluent comprehension, each experimental section includes an associated manuscript presented using the format and style of a scientific paper. Each experimental section is introduced by a summary of the framework and key concepts dealt with by each of the manuscripts, including a brief overview of the outcomes and their implications and a representative figure of including the main methods and/or results. Regardless, only the manuscripts contain all the complete methodology and set of outcomes. Accordingly, readers are strongly encouraged to read both. Note that the specific bibliography references of each experimental section can be found within the respective manuscripts.

After years of technical development and uses in causal brain mapping or diagnostic applications, noninvasive brain stimulation (NIBS) was rapidly adopted as for translational research tool in neurology and psychiatry and used to functionally explore contributions of cortical regions that once identified, were modulate for therapeutical purposes. Despite their highly promising potential, preclinical and clinical **outcomes employing repetitive TMS or tDCS have often been affected by a high degree of intraindividual and interindividual variability.** Such inconsistencies have ignited a relatively recent strategical shift towards an individualization of interventions informed by MRI based biophysical models of NIBS current distribution; and at the group level, towards the identification of predictive factors (either performance levels/clinical severity, anatomical and neurophysiological features) favoring cortical modulation and therapeutic efficiency.

In the first Experimental section of this PhD dissertation, we first present a published systematic review (Manuscript 1) and prospective vision and opinion piece focused on the efficacy of NIBS interventions for post-stroke voluntary motion disorders focusing on posture and gait, one of the most natural expressions of voluntary motion. In the manuscript, we identified the future challenges faced by the neurostimulation field to be in a position to exert significant clinical contributions in neurology and psychiatry. The systematic review explored the clinical efficacy of tDCS for the recovery of gait and posture impairments after stroke. To this end, we systematically examined an initial pool of 457 peer-reviewed clinical studies, exploring mechanistic models subtending recovery and in such framework we critically review the most adapted tDCS strategies to operate on different post-stroke physiological scenarios (early GABAergic inhibition subtending cell survival in acute stages, late sub-acute to chronic excitatory cascades promoting neurogenesis and circuit reorganization). A final pool of n=24 studies consisting in randomized- controlled trials, with a parallel-arms or a cross-over design, implementing double- or single-blinding schemes, with a sham or an active control group and a total sample size ranging from 8 to 81 participants. An extended detailed conceptual framework, analyses and discussions can be found in **Manuscript 1**.

Clinical efficacy varied substantially across studies, but tDCS strategies revealed a promising potential for clinical practice (Figure 25.A). More importantly, insights from clinical variability served to identify the ultimate factors and challenges driving successful interventions. A detailed examination of trial designs, allowed us to identify the following key challenges ahead to develop effective therapeutic strategies:

1. **The need for individualized stimulation protocols and adapted brain neurophysiological assessments**. Group-level assessments of post NIBS interventions are important to evaluate the general efficacy of an intervention and rule out the null hypothesis. Nevertheless, a large level of interindividual anatomical, patients baseline and lesion features require individualization of behavioral and neurophysiological deficits for adequate group comparisons. Importantly, brain-state or causal perturbation readouts can be used to individually understand the adaptive or maladaptive role of a brain circuit to a specific symptom and develop individualized stimulation strategies.

2. **The need of a better understanding of the neurophysiological mechanisms mediating plasticity and their guidance by NIBS approaches**. To improve our current understanding on the anatomical and neurophysiological contributions facilitating spontaneous or rehabilitation-induced recovery of motor functions is paramount for an effective management of these patients. Equally important is the detailed comprehension of the anatomical, neurophysiological and cognitive principles and determinants of NIBS action, particularly, for repetitive Transcranial Magnetic stimulation (TMS) or Transcranial Current Stimulation (tCS) in their different modalities.

3. **The need to implement stimulation strategies operating simultaneously in multiple cortical regions.** We ultimately aim at fostering higher levels of adaptive plasticity and functional remapping on perilesional areas by boosting the activity of distant interconnected regions with specific cognitive contributions able to facilitate the initially altered function. Indeed, stroke results in local damage with distant network mediated consequences (known as ‘diaschisis’ or ‘diaschetic’ effects) that prevent normal communication and synchronization. Whereas direct local perilesional systems can be modulated to promote effective remapping and reorganization, widespread interconnected structures can be considered for successful reorganization of resources as both regional and distant structures adapt to post-stroke environments.

Manuscript 1: *Corominas-Teruel, X., Mozo, R. M. S. S., Simó, M. F., Colomina Fosch, M. T., & Valero-Cabré, A. (2022). Transcranial direct current stimulation for gait recovery following stroke: A systematic review of current literature and beyond. Frontiers in Neurology, 13.* *https://www.frontiersin.org/articles/10.3389/fneur.2022.953939.*

A long list of relevant factors for the impact of neurostimulation approaches identified in the prior review paper are directly or indirectly addressed in the Experimental sections of this dissertation and the reader will have the opportunity to appreciate such work leading to a plan for effective individualization of NIBS interventions. Nonetheless, the fundamental technical challenge posed by a lack of spatial specificity of brain stimulation readouts when using concurrent NIBS and EEG recordings (notably via TMS-EEG), hence the need to rule out genuine direct effects of neurostimulation from later acting cortico- and subcortico-cortical reentry and peripheral inputs and identify the action site and gauge the magnitude of local effects in directly targeted cortical regions has been set as the first aim of this dissertation.

As indicated in the introduction, TMS-EEG allows for individualized, non-invasive monitoring of local and network activity with excellent temporal resolution at the millisecond scale). However, TMS-evoked EEG responses, known as Transcranial Evoked Potentials (TEPs) or TMS evoked potentials are spatially nonspecific, as they reflect the summation in time and space of the signals emitted by neurons in multiple cortical and subcortical regions, both directly and indirectly affected by a TMS pulse or pattern. Moreover, TMS-EEG datasets are generally analyzed at the sensor (electrode) level by selecting a constant group of EEG electrodes (under the TMS coil) in each individual of the same experimental cohort; and this is practiced despite the known fact that interindividual differences in head/brain anatomical features exert individually specific effects on the regional dispersion of electrical current fields (E-fields). Unfortunately, to day, no method can effectively limit such a lack of source specificity and the mismatch between a highly localized stimulated region (particularly when individualized MRI neuronavigation NIBS is employed) and the spatial ambiguity with regards to the sources of the emerging EEG signals. This situation challenges the reliability of existing analytical pipelines for TMS-EEG datasets precluding the direct exploration of EEG time courses and the extraction of biomarkers reflecting the primary effects of stimulation.

The spatial resolution of EEG sources is inherently limited due to the so-called *inverse problem*, which addresses the challenge of determining which could be the most reliable location(s), orientation(s), and magnitude(s) for cortical and subcortical brain source(s) that explains an observed pattern of topographical EEG activity (Figure 25.B). This question becomes an even bigger problem if one considers that there is not enough information on EEG data to estimate activity independently for a higher number of sources than the degrees of freedom available in EEG measurements constrained by the number of scalp EEG electrodes. Regardless, we can selectively extract activity from specific brain regions while suppressing others, using frameworks for source reconstruction and spatial filtering. To model and source-reconstruct EEG activity from brain, several methods exist. One can model the activity of preselected sources of interest from which we assume activity might have risen, by dipole fitting, assuming no other simultaneously active source in the brain. The same similar assumption is taken for the *beamformer* approaches, which at difference with the former relies on data covariance for source estimation. For TMS-EEG, evoked activity typically induces orthodromic and antidromic propagation across interconnected structures and the use of source reconstruction approaches assuming the absence of simultaneously active regions leads to misinterpretations. Distributed source models (like minimum norm estimators, MNE) used as a potential alternative to the former methods assume the presence of multiple simultaneous brain active sources, a scenario more compatible with the feature of TMS-EEG effects dependent on EEG source and head models. Additionally, the degree of leakage of a specific brain source can be evaluated by means of crosstalk functions (CTF), which describe the sensor sensitivity profile to a source compared to extracranial sensors (aka lead-field) and the degree of *leakage* by other sources into your estimate. Importantly, the CTF of a source or group of sources (within a region of interest, ROI), can be employed for spatial filtering while simultaneously weighting identified sources to minimize crosstalk by decreasing crosstalk from anywhere in the brain.

MRI neuronavigation offers precise information on the TMS coil position during TMS-EEG recordings, enabling individualized *post-hoc* modelling of the distribution of TMS-delivered currents (E-fields). Therefore, E-field prediction can serve to individually explore sources directly impacted by the stimulation and be used as prior assumptions

(aka '*priors*') to better isolate the direct primary effects of stimulation. In other words, by detecting and outlining the EEG sources impacted by stimulation according to an E-field distribution simulation model, crosstalk functions can be estimated and spatial filters applied to individually explore primary evoked activation of such regions of brain tissue. Accordingly, we here integrated on a common framework E-field simulations and EEG source reconstructions and implemented such an approach as a spatial filtering method intended to explore evoked activity into primarily targeted regions. A Minimum Norm-like Estimator (MNE) with special weighting constraints fulfilled by the subject-individual E-field simulation results was implemented to this end. Generally, first Finite Element Models (FEM) were reconstructed from individual MRI anatomical volumes. Next E-field simulations conducted on the basis of the real TMS coil positions employed in experimental sessions were computed. Finally, we created MNE-like and crosstalk functions and spatial filters focused on the sources directly impacted by stimulation.

Briefly, the pipeline can be formalized as follows: In EEG source estimation procedures, primary current activity generators are normally discretized into several voxels or triangulated (aka. the 'mesh' model) n sources. For each one, the signal that a source would produce on m sensors results in the linear forward vector (i.e., lead vector or topography) lj (m x 1, index j of n). Arranging all lj topographies into columns, the lead field matrix L is defined, and the signal y produced by an electrical current ($m\,X\,1$) results from the linear combination of the columns of L weighted by the s strengths of j sources:

$$y = Ls$$

Source reconstruction methods (aka. 'linear estimators') attempt to produce estimates of s. However, when the number of sources is larger than number of sensors, sources of no interest will leak into the estimate. The effect of all sources on our estimate can be described by applying a linear estimator w (i.e., spatial filter) to all vectors of L

$$CTFj = wjL$$

where, w can be formalized as:

$$w = (tL^T + (i - tL^T S^{-1} P)(P^T S^{-1} P)^{-1} P^T) S^{-1}$$

where P is a $m\,x\,p$ matrix with m sensors and p horizontally concatenated forward topographies, $i\ (1\,x\,p)$ is a 'stop-band' vector in source space per every discrete source p, $t\ (1\,x\,n)$ is a 'pass-band' vector in source space with n number of model sources and S is the noise term derived from $S = LL^T + \lambda^2 C$ where λ is the free regularization coefficient and $C\ (m\,x\,m)$ the covariance matrix. Assuming a lack of specific stop-bands and the aim to isolate signals from the NIBS targeted sources, while minimizing cross-talk from all other ones, a targeted jth column (i.e., source within the E-field boundaries) of $L(L_{.j})$, the ideal filter would have 0 anywhere except j locations where P would equal to $L_{.j}$ and tL^T would equal to $L_{.j}^T$ simplifying to

$$w = \frac{L_{.j}^T (LL^T + \lambda C_N)^{-1}}{L_{.j}^T (LL^T + \lambda C_N)^{-1} L_{.j}}$$

Finally, we experimentally tested the capabilities of our algorithm of decoupling individual-subject time-courses of the directly stimulated regions while minimizing the

contribution of distributed sources in the brain. In a cohort of n=18 stimulated healthy participants with single pulse TMS-EEG delivered to the left motor cortex, we verified that the here stated procedure and pipeline was effective reducing noise components emerging from source distributions differing from the primary stimulated source. Despite this pipeline was tested in TMS-EEG, the procedure can be easily adapted to support other NIBS modalities concurrent with EEG (such as tDCS, tACS, FUS, etc.) as well as the integration of DTI data. The details of this method and its experimental verification can be found in **Manuscript 2**. Complementary information has also been appended as supplementary materials in **Manuscript 3**.

Manuscript 2: *Corominas-Teruel, X., et. al., (2025). EPICURUS: E-field-based spatial filtering procedure for an accurate estimation of local EEG activity evoked by Transcranial Magnetic Stimulation*

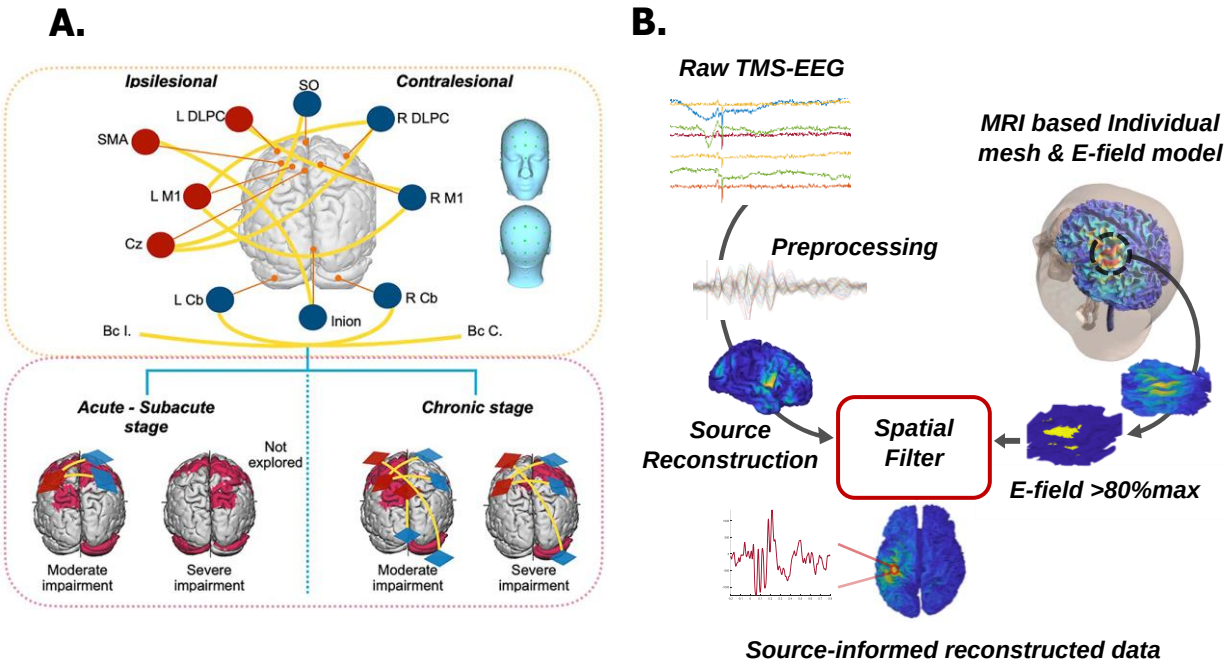


Figure 25. Scientific contributions contained in Manuscripts 1 & 2. A. Graphical illustration showcasing the network-based mechanistic rational of different brain stimulation regimes designed to enhance motor function in stroke patients. Manuscript 1 delves into the challenges and requirements of non-invasive brain stimulation (NIBS) within both experimental and clinical contexts, particularly focusing on the challenges to improve modulation efficacy following brain damage leading to motor impairment, with a focus on gait and posture. **B.** Schematic illustration of the major steps of the custom-programmed pipeline developed as part of this thesis to personalize procedures for more accurate analyses of concurrent TMS-EEG recordings. Manuscript 2 introduces and experimentally evaluates a computational pipeline implementing a TMS-EEG spatial filtering approach to increment reliability and personalize signal extraction restricted to the stimulation region. The approach operates as a minimum norm-like estimator and addresses the issue of the lack of spatial specificity affecting NIBS-EEG recordings concerning the primary stimulated regions.

4.1.1 Manuscript 1: Transcranial direct current stimulation for gait recovery following stroke: A systematic review of current literature and beyond. Frontiers in Neurology

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Transcranial direct current stimulation for gait recovery following stroke: A systematic review of current literature and beyond

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Background: Over the last decade, transcranial direct current stimulation (tDCS) has set promise contributing to post-stroke gait rehabilitation. Even so, results are still inconsistent due to low sample size, heterogeneity of samples, and tDCS design differences preventing comparability. Nonetheless, updated knowledge in post-stroke neurophysiology and stimulation technologies opens up opportunities to massively improve treatments.

Objective: The current systematic review aims to summarize the current state-of-the-art on the effects of tDCS applied to stroke subjects for gait rehabilitation, discuss tDCS strategies factoring individual subject profiles, and highlight new promising strategies.

Methods: MEDLINE, SCOPUS, CENTRAL, and CINAHL were searched for stroke randomized clinical trials using tDCS for the recovery of gait before 7 February 2022. In order to provide statistical support to the current review, we analyzed the achieved effect sizes and performed statistical comparisons.

Results: A total of 24 records were finally included in our review, totaling $n = 651$ subjects. Detailed analyses revealed $n = 4$ (17%) studies with large effect sizes (≥ 0.8), $n = 6$ (25%) studies with medium ones (≥ 0.5), and $n = 6$ (25%) studies yielding low effects sizes (≤ 0.2). Statistically significant negative correlations (rho $= -0.65$, $p = 0.04$) and differences ($p = 0.03$) argued in favor of tDCS interventions in the sub-acute phase. Finally, significant differences ($p = 0.03$) were argued in favor of a bifocal stimulation montage (anodal M1 ipsilesional and cathodal M1 contralesional) with respect to anodal ipsilesional M1.

Conclusion: Our systematic review highlights the potential of tDCS to contribute to gait recovery following stroke, although also the urgent need to improve current stimulation strategies and subject-customized

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interventions considering stroke severity, type or time-course, and the use of network-based multifocal stimulation approaches guided by computational biophysical modeling.

Systematic review registration: PROSPERO: CRD42021256347.

KEYWORDS

non-invasive brain stimulation (NIBS), transcranial direct cortical stimulation (tDCS), stroke, neurorehabilitation, neuromodulation, gait rehabilitation, biophysical modeling

Highlights

- A systematic review of tDCS studies in post-stroke gait rehabilitation was carried out.
- Differences in clinically relevant outcomes influenced by post-stroke delay at treatment onset and stimulation strategy are reported.
- Future tDCS interventions based on analyzing activity changes are able to predict motor recovery, optimized stimulation montages, parameters, and model-based customization ought to be explored.

Introduction

The rehabilitation of stroke sequels is among the major health problems modern societies are currently facing. Stroke stands out as the first cause of disability and the second cause of mortality in adults (1), with <20% of the affected subjects able to return to their prior professional and personal life. Although it has been classically presented as impacting old adults, stroke is becoming "*a disease of all ages*"also affecting young survivors, thereby further increasing its socioeconomic burden (2–4). Derived neurological deficits present a large heterogeneity often affecting multiple motor, sensory, and cognitive domains. The occurrence and prevalence of its symptoms are related to several variables such as the extent and location of the brain injury, subject's age, and pre-lesional neurological status (5, 6). The ability to recover close-to-normal or normal gait is one of the key symptoms in order to achieve adaptive reintegration to a functional community life. However, gait limitations are observed in more than 80% of stroke survivors (7), of which 25% will suffer from severe chronic and enduring deficits (8, 9).

Gait emerges from intrinsic excitatory-inhibitory balances within the cerebral and spinal central nervous system (CNS) and dynamic influences with the peripheral nervous system (PNS) and muscle groups. Each node, as part of a highly distributed cerebral motor network (M1, SMA, premotor cortex, cerebellum, etc.), contributes to a complex set of motor sub-processes (initiation, maintenance, error control as well as learning, planning, consolidation of human gait, etc.). Although usually compromised by the presence of hemiplegia, sensory and cognitive deficits, and walking abilities, which are much less dependent on cortical resources than fine motor skills, are often less impacted following cortical damage and cerebral-site-specific maladaptive plasticity (10). For this reason, in recent years, novel motor rehabilitation approaches (particularly non-invasive stimulation after stroke affecting the upper limbs) have attracted major interest. Unfortunately, non-motor cognitive deficits, in particular attentional and executive dysfunctions, can endure and prevent or slow down further motor rehabilitation process. For this reason, they would need to be specifically approached and facilitated (11–15).

Some well-identified functions of the healthy brain seem to be modulated through mutually inhibitory trans-callosal interhemispheric interactions able to maintain homeostatic excitatory/inhibitory balance in areas of the CNS. Roughly speaking, after a stroke, activity in the damaged hemisphere has been shown to decrease while contralateral homologous areas increase, further inhibiting the damaged hemisphere and preventing spontaneous remapping and recovery in perilesional areas sharing similar connectivity features and behavioral contributions (16). Accordingly, balance in the rivalrous transcallosal inhibitory drive has been positively associated with the degree of spared motor function and ultimately with recovery rates (17). Recovery can be achieved by directly increasing the excitability of perilesional regions in the damaged hemisphere; by suppressing homotopic spared regions of the contralesional hemisphere; the former and the latter simultaneously, or finally by activating ipsilateral spared cortical cerebral or cerebellar regions connected with peri-lesion areas. In such a scenario, non-invasive brain stimulation (NIBS) technologies can locally modulate activity in either peri-lesional or spared cortical areas of the lesioned or contralesional hemisphere, and rebalancing disbalanced networks has become a widely employed therapeutic strategy.

The framework and predictions posed by the interhemispheric mutually inhibitory interaction model, which placed the emphasis on the importance of networks linking spared and damaged areas, remain very influential when planning and assessing the coherence of neuromodulation

strategies in stroke subjects. This is particularly important in domains such as spatial attention and awareness deficits (hemispatial neglect), language deficits (aphasia), and upper limb motor paralysis. Nonetheless, novel approaches based on the identification from large databases of stroke cases of anatomical of functional determinants of spontaneous motor recovery are claiming increasing attention. Very particularly, structural connectivity-based models have identified areas linked by lesion-impacted white matter connectivity most frequently involved in the spontaneous recovery of upper limb function following damage, as a function of stroke baseline severity and phase locking (18). Most importantly, cortical regions that if adequately modulated through NIBS might be likely to drive functional recovery can be integrated when selecting the suitability of a stimulation strategy for the rehabilitation of stroke sequels.

Among different NIBS technologies, transcranial electrical stimulation (tES), which integrates transcranial direct current (tDCS), alternating current (tACS), and random noise (tRNS) stimulation, stands out thanks to its low cost, safety, and has set promise of synergistic impact when combined with conventional interventions (19, 20). To date, the clinical potential of tRNS is yet to be fully established in upper limb paralysis or gait rehabilitation. Differently, the effects of tACS have been evaluated in upper limb stroke rehabilitation (21) and shown to increase the strength of functional coupling between the primary motor cortex (M1) and the cerebellum at the beta and gamma bands (22, 23) in association with the control of muscle ankle activity in treadmill training (24). Nonetheless, tDCS remains the technological approach most widely evaluated for motor rehabilitation, and for this same reason, the focus of the current systematic review on gait recovery following stroke.

Transcranial DCS is based on the application of a low-intensity direct continuous current able to induce sub-threshold shifts of polarity-dependent membrane potentials and ensuing long-term potentiation/depression-like plasticity (25–27). Recent, meta-analyses addressed the impact of tDCS combined with other interventions on balance and postural control (28), gait and speed rehabilitation (29), and improvements in walking ability (30–32), and despite promising results, outcomes at the group level remain either heterogeneous or inconclusive. Such a disparity has been argued to emerge mainly from the large variety of post-ictal clinical presentations, baseline symptom severity, the influence of associated factors such as the presence of hemineglect, sensory or proprioceptive deficits, and the wide array of choices in tDCS parameters (cortical target, current intensity and density, electrode size, location, montages, etc.). A majority of tDCS trials have been carried out using the so-called "classic" bipolar electrode scheme based on a single anode and a single cathode delivering up to 2-mA peak intensity. Nonetheless, a move toward the use of multi-site set-ups targeting networks rather than isolated regions and a subject-customization of stimulation parameters guided by biophysical computational models and simulations of current distribution is slowly emerging in the field (33–35).

To contribute to this effort and ultimately improve motor rehabilitation following stroke, here we reviewed the state-of-the-art in the field by means of a systematic search and analysis of scientific literature reporting uses of tES, and particularly tDCS, for post-stroke gait impairments. On the finally selected sample of high-quality studies, we first assessed and quantified by recalculating effect sizes of the current success of tDCS strategies on gait rehabilitation following stroke; and second, we explored the influence of tDCS treatment intensiveness, periodicity, current intensity, and density, post-stroke time at tDCS treatment onset, and stimulation strategy on effect sizes across available outcome measures. On such a basis, we discussed the suitability of current tDCS strategies defined in terms of stimulation parameters and cortical targets used in the rehabilitation of gait following stroke. We finally discussed and speculated about novel tDCS targeting strategies, which take into account baseline severity and clinical progress pre-treatment onset, stroke lesion features, and individual subject's anatomy and brain features through biophysical computational models might help profile more efficient interventions.

Materials and methods

The current systematic review is presented according to the recommendations in the Preferred Reporting Items for Systematic Review and Meta-analyses: The PRISMA Statement (36). The protocol and systematic review were prospectively registered on June 2021 through PROSPERO: CRD42021256347.

Data sources and search strategy

The previous literature search focused on stroke studies that used tDCS for the recovery of functional balance and walking, was conducted on 7 February 2022 using web-based databases including MEDLINE (through PubMed), SCOPUS, Cochrane Central Register of Controlled Trials (CENTRAL), and CINAHL (through EBSCOhost). Keywords with regard to the intervention, domain, and condition included the following: ("tES" OR "transcranial electrical stimulation" OR "tDCS" OR "transcranial direct current stimulation" OR "anod*" OR "cathod*") AND ("balance" OR "posture" OR "gait" OR "walk" OR "locomotion") AND ("stroke" OR "cerebrovascular accident" OR "brain ischemia"). Reference lists were manually screened by two independent expert observers to be identified by common agreement relevant studies.

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Study eligibility criteria

Studies were included when the following criteria were met: (1) application of tES in human subjects with stroke of at least 18 years of age; (2) assessment of the effect of tES on the recovery of walk and/or balance including assessments of these parameters; (3) comparison of an active tES alone or combined with other rehabilitation approaches (excluding however other brain stimulation tools in combined therapeutic approaches); (4) randomized-controlled trials, including crossover and parallel designs; and (5) peer-reviewed articles published in English before 7 February 2022. Studies were excluded if the retrieved item (1) was a review study, a single case report, an editorial comment, or a meta-analysis of prior studies; (2) included stroke subjects with a prior history of other neurological or musculoskeletal diseases; (3) included healthy subjects or subjects with other neurological or musculoskeletal diseases as control groups; (4) consisted of abstracts with no associated full article published in a peer-reviewed English-speaking journal.

Study selection and data extraction

Authors XC-T, AV-C, and MC independently screened the retrieved studies by carefully reading titles and abstracts. Once a final agreement between reviewers was achieved, the following information was extracted from each article: (1) author/s and year of publication; (2) study design, demographics, and clinical characteristics; (3) total number of enrolled participants, number of participants included/completed the study, and number of participants in intervention/control group; (4) subject's age; (5) subject's gender; (6) subject's stroke type and baseline severity; (7) subject's stroke phase; (8) intervention protocol design; (9) stimulated targets; (10) methods used to localize cortical targets; (11) stimulation current intensity; (12) electrode size and current density; (13) total session number; (14) duration; (15) periodicity; (16) associated interventions performed on the experimental or control group during stimulation regimes; (17) outcome measures used to report the results; and (18) effect sizes. Data were extracted using a structured table (refer to summary in Table 1).

Beyond a conventional systematic review and meta-analysis on gait rehabilitation (28–32), we here linked the study outcomes with potential factors that may be bearing on gait rehabilitation. To this end, we focused on characterizing functional improvements of gait *velocity*, *endurance*, and *stability/mobility* in post-stroke subjects evaluated through at least one of the following clinical tests or kinetic assessments: the 10-meter walk test (10MWT), the six-minute walking test (6mWT), the Timed Up and Go Test (TUG), Tinetti-POMA (T-POMA), and the Berg Balance Scale (BBS). We assessed potential relationships with maximum effect sizes across available tasks, 24–72 h after post-tDCS treatment and treatment intensiveness (total number of sessions), and periodicity (number of sessions per week). We also assessed the relationship between effect sizes and tDCS current intensity and density. Moreover, we assessed the efficacy of tDCS interventions in relation to variables informing indirectly on post-stroke plasticity events such as time post-stroke event at treatment onset. Finally, after the inspection of the stimulation strategies used in the included studies, retained items were divided according to their stimulation strategy based on electrode location in order to assess potential relationships with effect sizes and stimulation strategies.

Quality assessment of individual studies

Since our study focused on the modulation of motor functionality, the Physiotherapy Evidence Database (PEDro) was used to conduct study quality assessments. The PEDro scale has been proven valid and reliable (61) to evaluate the methodological quality of a set of eligible studies (62), based on the Delphi List criteria (63). It is based on the same items as the Cochrane Risk of Bias tool (CRBt) to assess the methodological quality of a clinical study, in which one point is given for each satisfied criterion. Since, item 1, which assesses external validity, is not used to calculate the PEDro total score, the final scores range between 0 and 10. Studies are considered to be of excellent quality (scores equal to or higher than 9/10), good quality (between 6 and 8/10), fair quality (between 4 and 5/10), and poor quality (lower or equal to 3/10).

Statistical analysis

In order to understand the effect of tDCS to facilitate gait recovery in post-stroke subjects compared to other rehabilitation strategies (i.e., physical exercise, robot-assisted gait therapy, etc.), we have conducted a series of statistical comparisons exploring clinical outcome improvement after tDCS interventions taking into account the most relevant parameters linked to different tDCS strategies. First, effect sizes (Cohen's *d*) were re-calculated using the outcomes of the earliest post-treatment evaluation (usually for all studies within 24–72 h after completing the treatment), on the 6mWT to assess gait *endurance*, the 10MWT, and/or kinetic tests to explore gait *velocity*, and TUG and/or T-POMA and/or BBS for the evaluation of gait *stability/mobility*. This specific post-tDCS treatment evaluation time was selected to avoid any influence of the last tDCS session. Moreover, 24–72 h after completing the treatment was the milestone most often reported between the selected publications (16 of 24), hence allowing the fairest comparison across studies. Estimations of the effect size at ulterior post-tDCS follow-up points failed due to the lack of

TABLE 1 Summary of included data by each of the 24 retained individual studies.

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Andrade et al. (37)	RCT parallel, double blinded, sham controlled	I vs. F: 60/60 Ig vs. Cg: 15/15/15/15 Age: 69 ± 3.2 M vs. W: 33/25	60I Acute-subacute Medium	Group A: A-M1 ipsilesional C-supraorbital contralesional Group B: A-M1 ipsilesional C-M1 contralesional Group C: A supraorbital ipsilesional C-M1 contralesional 10/20 EEG system and anatomical limits	2mA, 5 × 7 saline-soaked sponge 0.05mA/cm^2 10 sessions, duration of each not reported 5 days a week × 2 weeks Intervention group: A-tDCS, C-tDCS, Bilateral tDCS + conventional physical therapy Control group(s): Sham tDCS + conventional physical therapy Concurrent rehabilitation 3h a week × 2 weeks during tDCS treatment	FSST OSI FES-I BBS 6mWT STS	BBs: Insufficient data 6MWT: Insufficient data
Cattagni et al. (38)	RCT cross-over, double blinded, sham controlled	I vs. F: 24/24 Ig vs. Cg: 12/12 Age: 57 ± 13 M vs. W: 19/5	17I/7H Chronic Mild-medium	A-leg area of the ipsilesional motor cortex, laterally to Cz C-supraorbital contralesional 10/20 EEG system and anatomical limits	2mA, 5 × 7 saline-soaked sponge 0.06mA/cm^2 2 sessions (1 sham), 30 min each 1 a week × 2 weeks (7 days wash out) Intervention group: A tDCS in rest Control group(s): Sham tDCS in rest No concurrent rehabilitation program nor online rehabilitation during tDCS	3D gait analysis EMG activity of the RF, GM, SOL and TA muscles BDNF genotype	Gait velocity (kinetic assessment): 0.07

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Chang et al. (39)	RCT parallel, double blinded, sham controlled	I vs. F: 24/24 Ig vs. Cg: 12/12 Age: 62.8 ± 10.4 M vs. W: 15/9	24I Acute-subacute Medium	A-TA area of the ipsilesional motor cortex C- supraorbital contralesional Hotspot detected with TMS MEP	2mA, 3 to 6 cm diameter saline-soaked sponge A 0.28 mA/cm^2 and C 0.07 mA/cm^2 10 sessions, 10 min each 5 a week × 2 weeks Intervention group: A-tDCS + conventional intensive physical therapy Control group(s): Sham tDCS + conventional intensive physical therapy Concurrent rehabilitation program 2.5 h daily, 6 days a week × 2 weeks + online rehabilitation during each tDCS session	MEP of the TA FMA-LE MI-LE FAC BBs	BBs: 0.3 Gait velocity: 0.2
Danzl et al. (40)	RCT parallel, double blinded, sham controlled	I vs. F: 8/8 Ig vs. Cg: 6/2 Age: 67.8 ± 11.7 M vs. W: 4/4	5I/3H Chronic Medium-severe	A-center of leg area (cz) C-supraorbital area	2mA, 5 × 5 and 5 × 7 saline-soaked sponge A 0.08mA/cm^2 C 0.05mA/cm^2 12 sessions, 20 min each 3 a week × 4 weeks Intervention group: A-tDCS + lokomat Control group(s): Sham tDCS + lokomat Concurrent rehabilitation program, 3 h a week × 4 weeks, and rehabilitation following each tDCS session	10 MWT TUG BBs FAC SIS-16	10 MWT: Insufficient data TUG: Insufficient data BBs: Insufficient data

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Fusco et al. (41)	RCT parallel double blinded, sham controlled	I vs. F: 14/11 Ig vs. Cg:7/7 Ag: 58.36 ⊥ 14.3 M vs. W: 5/5	11I Subacute Mild-severe	A-Ipsilesional shoulder C-Contralesional M1 10/20 EEG system	1.5mA, 5 × 7 saline-soaked sponge 0.04mA/cm^2 10 sessions, 10 min each 5 a week × 2 weeks Intervention group: C-tDCS + intense conventional physical therapy Control group(s): sham tDCS + intense conventional physical therapy Concurrent rehabilitation program, 1.5h daily, 5 days a week × 2 weeks following tDCS sessions	10MWT TUG 6mWT 9HPT Dynamometer FMA-UE CNS RMI BI FAC	10MWT: Insufficient data TUG: Insufficient data 6mWT: Insufficient data
Geroin et al. (42)	RCT parallel, double blinded, sham controlled	I vs. F: 30/30 Ig vs. Cg:10/10/10 Ag: 62.7 ± 6.4 M vs. W:23/7	30I Chronic Medium	A-Ipsilesional leg area C-Supraorbital contralesional	1.5mA, 7 × 5 saline-soaked sponge, 0.04mA/cm^2 10 sessions,7 min each 5 a week × 2 weeks Intervention group: tDCS + RAGT, overground walking Control group(s): sham tDCS + RAGT Concurrent rehabilitation program, 50 min daily, 5 days a week × 2 weeks + online rehabilitation during each tDCS session	6mWT 10MWT Kinetic evaluation FAC RMI Ashworth MI leg	6mWT:0.3 10MWT:0.18
Kindred et al. (43)	RCT parallel, double blinded, sham controlled	I vs. F: 21/18 Ig vs. Cg: 7/7/7 Ag: 64.8 ± 12.5 M vs. W: Not reported	Not reported Chronic Medium	Group A: HD 4 × 1 montage with the A-M1 ipsilesional Group B_ HD 4 × 1 montage with the C-M1 ipsilesional 10/20 EEG system + computational modeling for E-field prediction	2mA, 1cm diameter, A 0.63mA/cm^2 C 0.15mA/cm^2 1 session, 20 min each 1 day Intervention group: A-tDCS, C tDCS + ergometer pedaling Control group(s): Sham tDCS + ergometer pedaling No Concurrent rehabilitation program but online rehabilitation during each tDCS session	Gait kinematics Ground reaction forces over ground walking Corticomotor TA response MEP of the TA	Gait velocity (kinetic assessment): insufficient data

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Klomjai et al. (44)	RCT- cross over, double blinded, sham controlled	I vs. F: 19/19 Ig vs. Cg: 19/19 Ag: 57.2 ⊥ 2.8 M vs. W: 14/5	19I Subacute Medium	A-M1 ipsilesional C-M1 contralesional 10/20 EEG System and anatomical limits	2mA, 7 × 5 saline-soaked sponge 0.05mA/cm^2 2 sessions (1 sham), 20 min each 1 a week × 2 weeks (7 days wash out) Intervention group: bilateral TDCS + conventional physical therapy Control group(s): Sham tDCS + conventional physical therapy No concurrent rehabilitation program, but 1 h of rehabilitation following each tDCS session	TUG Five times STS Peak knee torque of extension	TUG: 0.4
Leon et al. (45)	RCT parallel, single blind, active control	I vs. F: 50/49 Ig vs. Cg: 9/17/23 Ag: 48 ⊥ 11 M vs. W: 34/15	29I/21I Subacute Severe	Group A: A-Cz vertex C-right supraorbital area Group B: A-M1 ipsilesional C-Supraorbital contralesional 10/20 EEG system	2mA, 7 × 5 saline-soaked sponge 0.05mA/cm^2 20 sessions, 20 min each 5 days a week × 4 weeks Intervention group: A-leg tDCS, A-hand tDCS + daily RAGT and a complete rehabilitation intense program Control group(s): daily RAGT and a complete rehabilitation intense program Concurrent rehabilitation program,5h daily, 5 days a week × 4 weeks + online rehabilitation during each tDCS session	10MWT FAC	Group A : 10MWT:0.03 Group B: 10MWT:0.19

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'd
Liang et al. (46)	RCT cross-over, double blinded, sham controlled	I vs. F: 10/10 Ig vs. Cg: 10/10 Age: 58.9 ± 9.5 M vs. W: Not reported	Not reported Chronic Medium	A-M1 ipsilesional, 1 cm anterior to the cranial vertex C-Supraorbital contralesional 10/20 EEG system and anatomical limits	2mA, 5 × 5 saline-soaked sponge 0.08mA/cm^2 2 sessions (1 sham), 20 min each 1 day every 2 weeks (14 days wash out) Intervention group: A-tDCS + center of gravity shift training in a force platform Control group(s): Sham tDCS + center of gravity shift training in a force platform No concurrent rehabilitation program, but online rehabilitation during each tDCS session	BBs Forward Reach Test 10MWT Bertec Advantage Computerized dynamic posturography	BBS:0.12 10MWT:0.23
Madhavan et al. (47)	RCT parallel, double blinded, sham controlled	I vs. F: 81/81 Ig vs. Cg: 20/20/21/20 Age: 58.7 ± 9.7 M vs. W:55/26	53I/28I1 Chronic Medium	A-M1leg ipsilesional C-Supraorbital contralesional Hotspot detected with TMS MEP	1mA, 5 × 2.5 and 4.5 × 5.5 saline-soaked sponge, A 0.08 mA/cm^2 C 0.04 mA/cm^2 12 sessions, 15 min each 3 a week × 4 weeks Intervention group: tDCS + AMT + HISTT, AMT + HIST, tDCS + HIST Control group(s): sham tDCS + HISTT Concurrent rehabilitation program, 3h a week × 4 weeks, prior to rehabilitation	10MWT CME 6mWT BBs SIS-16 TUG miniBESTest FMA-LE	10MWT: 0.39 6mWT:0.28 BBs:0.12 TUG:0

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'd
ji et al. (48)	RCT cross-over, double blinded, sham controlled	I vs. F: 30/30 Ig vs. Cg: 30/30 Age: 62.9 ± 10.5 M vs. W: 21/9	17I/13H Subacute Medium	A-SMA ,3.5 cm anterior to Cz C-over the inion 10/20 EEG system	1mA, 5 × 5 saline-soaked sponge 0.04mA/cm^2 10 (5 sham) sessions, 20 min each 5 a week × 2 weeks (3 days wash out) Intervention group: A-tDCS + body weight-supported treadmill gait training Control group(s): Sham tDCS + body weight-supported treadmill gait training Concurrent rehabilitation program, 1 h daily, 5 days a week × 2 weeks + online rehabilitation during each tDCS session	10MWT TUG FMA-LE Tinetti-POMA Trunk Impairment Scale	10MWT:0.42 TUG:0.04 T-POMA:0.7
Mitsutake et al. (49)	RCT parallel, single blind, sham controlled	I vs. F: 34/31 Ig vs. Cg: 17/11/11 Age: 72.6 ⊥ 10.7 M vs. W: 19/15	28I/6H Subacute Medium	A-leg area of the ipsilesional cortex, lateral to Cz C- Supraorbital contralesional 10/20 EEG system	2mA, 5 × 5 saline-soaked sponge 0.05mA/cm^2 7 sessions, 20 min each Daily for a week Intervention group: A-tDCS + conventional rehabilitative intervention, A-tDCS + Functional electrical stimulation of the peroneal nerve and conventional rehabilitative intervention Control group(s): Functional electrical stimulation of the peroneal nerve and conventional rehabilitative intervention Concurrent rehabilitation program, 1h for 7 consecutive days + online rehabilitation during each tDCS session	10MWT Trunk acceleration	10MWT:0.4

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/ finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/ baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Ojardias et al. (50)	RCT cross-over, double blinded, sham controlled	I vs. F: 20/18 Ig vs. Cg: 18/18 Age: 57.4 ± 3.6 M vs. W: 12/6	15I/31I Chronic Medium	A-M1 ipsilesional, over the leg area C-Supraorbital contralesional Hotspot localized with TMS MEP and magnetic resonance	2mA, 5 × 5 saline-soaked sponge 0.08mA/cm^2 2 sessions (1 sham), 20 min each 1 day a week × 2 weeks (11 days wash out) Intervention group: A-tDCS in rest Control group(s): Sham tDCS in rest No concurrent rehabilitation program nor online rehabilitation during tDCS sessions	Wade test 6mWT Step length and symmetry Balance in center of pressure platform	6mWT: insufficient data
Park et al. (51)	RCT parallel, not reported, sham controlled	I vs. F: 24/24 Ig vs. Cg: 8/8/8 Age: 59.4 ± 10.6 M vs. W: Not reported	16I/8H Chronic Mild–medium	A-Over Cz area of the left parietal lobe C-Right supraorbital area 10/20 EEG system	2mA, sponge size not reported 12 sessions, 15 min each 3 days a week × 4 weeks Intervention group: A-tDCS + TRT, TRT alone Control group(s): Sham tDCS + TRT Concurrent rehabilitation program, 30 min. daily, 3 days a week × 4 weeks + online rehabilitation during each tDCS session.	Kinematic evaluation of gait (velocity, stance phase, swing phase and step length)	Gait velocity (kinetic assessment): 0.03
Picelli et al. (52)	RCT parallel, double blinded, sham controlled	I vs. F: 30/30 Ig vs. Cg: 10/10/10 Age: 62.8 ± 8.3 M vs. W: 22/8	30I Chronic Medium-severe	A-M1 ipsilesional C- Supraorbital contralesional 10/20 EEG system	2mA, 7 × 5 saline-soaked sponge 0.05mA/cm^2 10 sessions, 20 min each 5 days a week × 2 weeks Intervention group: A-tDCS + tsDCS +RAGT, A-tDCS + sham tsDCS + RAGT Control group(s): Sham tDCS + tsDCS + RAGT Concurrent rehabilitation program, 1h daily 5 days a week × 2 weeks + online.	6mWT FAC MI-LE AS Spatiotemporal gait parameters	6mWT:0.28

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/ finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/ baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
Picelli et al. (53)	RCT parallel, single blind, active control	I vs. F: 20/20 Ig vs. Cg: 10/10 Age: 62.7 ± 10 M vs. W: 13/7	20I Chronic Medium-severe	Group A: A- Buccinator contralesional C-Cerebellum contralesional Group B: A-M1 ipsilesional C-Supraorbital contralesional 10/20 EEG system	2mA, 4cm diameter saline-soaked sponge 0.15 mA/cm^2 10 sessions, 20 min each rehabilitation during each tDCS session 5 days a week × 2 weeks Intervention group: c-tcDCS + tsDCS + RAGT Control group(s): A tDCS + tsDCS +RAGT Concurrent rehabilitation program, 1 h daily, 5 days a week × 2 weeks + online rehabilitation during each tDCS session	6mWT FAC MI-LE AS Spatiotemporal gait parameters	6mWT: Insufficient data
Picelli et al. (54)	RCT parallel, single blind, active control	I vs. F: 40/39 Ig vs. Cg: 20/19 Age: 64.6 ± 10.1 M vs. W: 23/17	40I Chronic Medium-severe	Group A: A- Buccinator contralesional C- contralesional cerebellum Group B: A-Buccinator ipsilesional C-ipsilesional cerebellum 10/20 EEG system	2mA, 4 cm diameter saline-soaked sponge 0.15 mA/cm^2 10 sessions, 20 min each 5 days a week × 2 weeks Intervention group: c-tcDCS ipsilesional − tsDCS + RAGT Control group(s):c-tcDCS contralesional + tsDCS + RAGT Concurrent rehabilitation program, 1 h daily, 5 days a week × 2 weeks + online rehabilitation during each tDCS session	6mWT FAC MI-LE AS Spatiotemporal gait parameters	6mWT: Insufficient data

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/ finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/ baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'd
Prathum et al. (55)	RCT parallel, double blinded, sham controlled	I vs. F: 26/24 Ig vs. Cg: 12/12 Ag: 57.75 ± 2.45 M vs. W: 18/9	24I Chronic Medium	A-M1 ipsilesional C-M1 contralesional 10/20 EEG system	2mA, 7 × 5 saline-soaked sponge 0.05mA/cm^2 12 sessions, 20 min each 3 days a week × 4 weeks Intervention group A-tDCS + Home-based exercise program Control group(s): Sham tDCS + Home-based exercise program Concurrent rehabilitation program, 1h daily 3 days a week × 4 weeks. tDCS sessions performed prior to rehabilitation	FMA Wolf Motor Function TUG Five times STS 6mWT Muscle strength assessment	TUG:0.4 6mWT:1.1
Saeys et al. (56)	RCT cross-over, double blinded, sham controlled	I vs. F: 31/31 Ig vs. Cg: 31/31 Ag: 63.2 ± 8.42 M vs. W: 17/14	29I/5H Subacute Medium	A-M1 ipsilesional C-M1 contralesional 10/20 EEG system	1.5mA, 7 × 5 saline-soaked sponge 0.04mA/cm^2 32 sessions (16 sham), 20 min each 4 days a week × 4 weeks Intervention group: A tDCS + multidisciplinary intense physical and occupational therapy Control group(s): Sham tDCS + multidisciplinary intense physical and occupational therapy Concurrent rehabilitation program, 1h daily, 4 days a week × 4 weeks. tDCS sessions performed prior to rehabilitation	Tinetti-POMA RMI Trunk Impairment Scale	T-POMA: 0.94

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/ finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/ baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'd
Seo et al. (57)	RCT parallel, double blinded, sham controlled	I vs. F: 21/17 Ig vs. Cg: 8/9 Ag: 62 ± 8.9 M vs. W: 16/5	16I/5H Chronic Medium	A-leg area ipsilesional, lateral to the C2 position C-Supraorbital contralesional 10/20 EEG system	2mA, 7 × 5 saline-soaked sponge 0.05mA/cm^2 10 sessions, 20 min each 5 days a week × 2 weeks Intervention group: A-tDCS + RAGT Control group(s): Sham TDCS + RAGT Concurrent rehabilitation program, 1h daily, 5 days a week × 2 weeks. tDCS sessions performed prior to rehabilitation	FAC 10MWT 6mWT BBs FMA LE Medical Research Council scale MEP of abductor hallucis	10MWT:1.33 6mWT:0.6 BBs:0.2
Talitis et al. (58)	RCT parallel, double blinded, sham controlled	I vs. F: 14/14 Ig vs. Cg: 7/7 Ag: 61.8 ⊥ 12 M vs. W:11/3	14I Subacute Medium	A-M1 ipsilesional C-M1 contralesional 10/20 EEG system	2mA, 3 × 5 saline-soaked sponge 0.08mA/cm^2 1session, 15 min 1 day Intervention group: bifocal tDCS in resting state Control group(s): sham tDCS in resting state No concurrent rehabilitation program nor online rehabilitation during tDCS sessions	Tinetti-POMA TUG	T-POMA:1.21 TUG:0.52
Utarapichat et al. (59)	RCT cross-over, single blind, sham controlled	I vs. F: 10/10 Ig vs. Cg: 5/5 Ag: 57.1 ⊥ 12.2 M vs. W: 6/4	10I Chronic Medium	A-Ipsilesional leg motor area C-Supraorbital contralesional 10/20 EEG system	2mA, 3 × 3 and 5 × 5 saline-soaked sponge A 0.22mA/cm^2 C 0.08mA/cm^2 2 sessions (1sham), 10 min each 2 days a week (48 h wash-out) Intervention group: A tDCS in rest Control group(s): sham tDCS in rest No concurrent rehabilitation program nor online rehabilitation during tDCS sessions	Root mean square amplitude and median frequency of vastus medialis and TA TUG	TUG:0.04

(Continued)

TABLE 1 (Continued)

References	Design	Subjects N (included/finished; intervention group/control group; age; man/woman)	Stroke demographics (type/phase/baseline impairment)	Target areas and electrode positions (anodal vs. cathodal), tools used to allocate targets	tDCS protocol (intensity & periodicity, controls and associated interventions)	Outcome measures	Cohens'*d*
van Asseldonk et al. (60)	RCT cross-over, double blinded, sham controlled	I vs. I': 10/10 Ig vs. Cg: 10/10 Age: 58 ± 11 M vs. W: 4/6	8I/2H Chronic Medium	Group A: A-M1 ipsilesional C-Supraorbital contralesional Group B: A-M1 ipsilesional C-M1 contralesional Hotspot localized with TMS MEP and anatomical limits	2mA, 5 × 7 saline-soaked sponge 0.05mA/cm^2 3 sessions, 10 min each 1 day a week × 3 weeks (7 days wash out) Intervention group: A-tDCS, bifocal tDCS in rest Control group(s): sham tDCS in rest No concurrent rehabilitation program nor online rehabilitation during tDCS sessions	Kinematic assessment	Gait velocity (kinetic assessment): Insufficient data

A, anodal; AMT, ankle motor tracking; AS, Ashworth scale; BBs, berg balance scale; BDNF, brain derived neurotrophic factor; BI, Barthel index; C, cathodal; CME, corticomotor excitability; CNC, Canadian neurological scale; EMG, electromyography; FAC, functional ambulatory categories; FES-I, the falls efficacy scale International; FSST, four square step test; FMA, fulg Meyer assessment; FMA-LE, fulg Meyer assessment lower extremity; FMA-UE, fulg Meyer assessment upper extremity; GM, gastrocnemius medialis; HSTT, high-intensity speed-based treadmill training; I, ischémic; Live rhb, a rehabilitation program runs in parallel with the intervention; MEP, motor-evoked potential; MI, motricity index; MI-LE, motricity index lower extremity; M1, primary motor area; OSI, overall stability index; Online rhb, tDCS is applied in the same session simultaneously with rehabilitation; RAGT, robot assisted gait training; RF, rectus femoralis; RMI, Rivermead mobility index; SMA, supplementary motor area; SIS-16, stroke impact scale 16; SOL, soleus; STS, sit to stand test; TA, anterior tibial; TMS, transcranial magnetic stimulation; TRT, task related training; tsDCS, trans-spinal direct current stimulation; TUG, time up and go test; 6mWT, six minute walking test; 9HPT, nine hold peg test; 10MWT, ten meter walking test.
The acute phase was considered within the first 2 weeks after stroke, the subacute phase between the 2nd and the 24th week, and the chronic phase more than 24 weeks after the stroke. Classification of the baseline impairment (mild, medium, and severe) was based on the inclusion criteria of the studies and the comprehension of the baseline assessment results.

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Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation to improve balance and gait ability in the rehabilitation of stroke patients
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available data in a consistent number of studies (only five provided data for accurate calculations hindering comparison).

Second, Spearman's rank-order correlation coefficient was used to explore possible interactions between tES achieved recovery effects (effect size) in *endurance*, *velocity*, and *stability/mobility* over the studies and treatment *intensiveness*, *periodicity*, tDCS current *intensity*, tDCS current *density*, and *time since stroke* episode. To better understand the purpose of the effect, multiple regressions were complementarily conducted. Then, studies were grouped and categorized by their characteristics [treatment *intensiveness* (N sessions total, categorized into four groups: 1–2 sessions, 3–7 sessions, 8–12 sessions, and +12 sessions), *periodicity* (N sessions × week, categorized into six groups: 1 session every 2 weeks, 1 session × week, 2 sessions × week, 3 sessions × week, 4 sessions × week, and 5 sessions × week), tDCS current *intensity* (categorized into three groups: 1mA, 1.5mA, and 2mA), current *density* (categorized into three groups: $\leq$0.05, 0.05 $\leq$ 0.08, and 5), *time since stroke episode* (N weeks post stroke, categorized into four groups: from 0 to 2 weeks, from 0 to 24 weeks, from 2 to 24 weeks, and + 24 weeks), and *stimulation strategy* (categorized into four groups: anodal ipsilesional M1 or SMA or leg area tDCS and contralateral supraorbital or prefrontal or inion return, cathodal contralesional M1 tDCS and ipsilesional supraorbital return, dual anodal, and cathodal M1 bilateral stimulation, cerebellar cathodal stimulation)]. After the categorization, data were tested with the Shapiro–Wilk test revealing a non-normal distribution. Then, the one-way Kruskal–Wallis test was used to analyze if there are any differences between groups within every characteristic regarding effect sizes. Only in case, the Kruskal–Wallis yielded statistically significant differences, and *post-hoc* analyses corrected for multiple comparisons using the Bonferroni method were performed. Statistical significance was set at $p < 0.05$. Note that for the *stability/mobility* outcome measures, which could be potentially represented by up to three clinical tests, when more than one test was available; statistical comparisons were computed on measures based on the mean value of the reported outcomes (nonetheless, notice this was the case of only two of the 24 studies, refer Table 1). Finally, significant results were represented in boxplots. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, v.25.0, IBM, USA).

Results

This systematic review includes items published before 7 February 2022. After the removal of duplicates and articles that did not meet selection criteria, a total of 24 controlled randomized (of which eight crossovers and 16 parallel/five single-blinded and 19 double-blinded) trials were included, totaling 651 subjects (37–60). The PRISMA 2020 flow diagram of the search process is shown in Figure 1.

Study selection and characteristics

A total of 457 studies were identified through the search in the four above-mentioned databases (from now on also referred to as "items"). After removing the duplicates and screening the titles and abstracts, 81 items (18%) were retained for full-text analysis by our two expert reviewers, of which 57 (70%) were excluded because they failed to meet inclusion/exclusion criteria. Of the 57 excluded items, 17 (30%) studies did not focus on the recovery of walk and/or balance neither included assessments of these parameters, six (11%) were not randomized-controlled trials, 4 (7%) included stroke subjects with a prior history of other neurological or musculoskeletal diseases, one (2%) included healthy subjects as control group lacking a sham parallel group, 24 (42%) items were not backed up by a full article published in a peer-reviewed English journal, one (2%) item was not written in English, and four (7%) worked in animal stroke models (Figure 1). All the 24 included studies were randomized-controlled trials, 16 (67%) used a parallel design, 8 (33%) a cross-over design, 19 (79%) implemented a double-blinded scheme, and 5 (21%) a single-blinded scheme; 21 (87%) had a sham control group and 3 (12%) used an active control group. The sample size ranged from 8 to 81 participants. Characteristics of the included studies are summarized in Table 1.

Quality assessment

PEDro scores of the included 24 studies ranged from 6 to 10 and the mean quality of the studies reached a qualification of "good"; the average score for analyzed articles was 7/10. Besides, six items (25%) reached scores deemed of "excellent" quality and 18 items (33%) were considered of "good" quality. All studies specified eligibility criteria. The full PEDro assessment is shown in Table 2.

Subjects: Demographic and individual considerations

Taken together, all studies included a total of 651 subjects of both genders (383 men and 213 women), gender remained unclear or unreported for 55 subjects. The mean average age was 60 years; 491 subjects had an ischemic stroke; 129 subjects were reported as having suffered a hemorrhagic stroke, whereas stroke type remained unspecified for 31 subjects. It should be noted that for most of the participants, the stroke episode was the first-ever ischemic event. Regarding time post-stroke, nine studies (37%) included subjects recruited in the acute-subacute

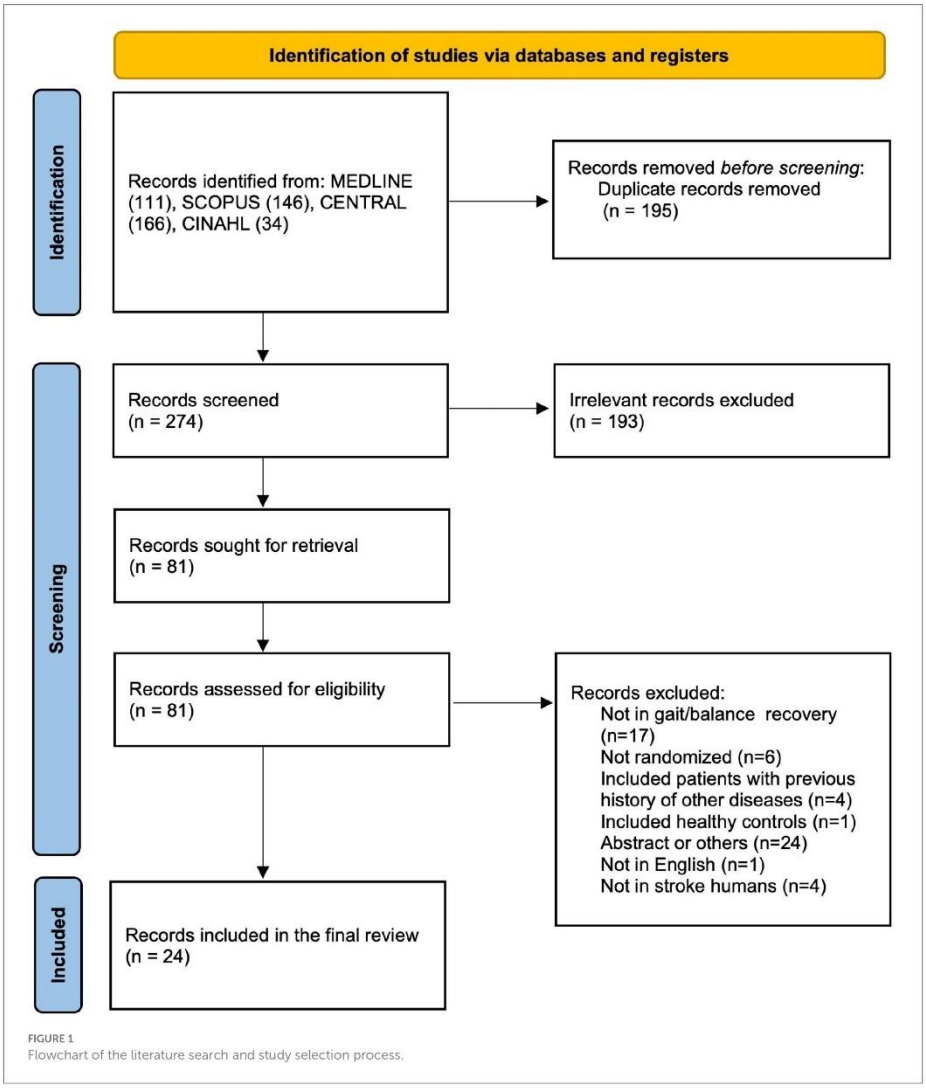


FIGURE 1
Flowchart of the literature search and study selection process.

phase and 15 (62%) at the chronic stage. The 24 retained studies were classified into three different levels (mild, medium, and severe) according to the severity of post-stroke impairment at baseline of their cases and their pre-defined severity inclusion criteria. In that context, two studies (8%) included subjects with a mild-medium baseline impairment, 14 (58%) subjects with a medium baseline impairment, 6 (25%) subjects with a medium-severe baseline impairment, whereas 1 (4%) and 2 (8%) studies included, respectively, only severe or mild-to-severe subject cases.

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TDCS methods

All 24 studies (100%) aimed to manipulate a single cortical target either excitatorily or inhibitorily, through the application of tDCS using saline-soaked sponges of different sizes. The vast majority of the selected studies, 23 out of 24 (96%), used bipolar montages (i.e., one anode and one cathode), with the exception of a single study (4%) (43), that used a high-density stimulation montage (4 × 1 HD-tDCS system). In the same vein, the vast majority of reports, 22 out of 24 (91%), based their interventions on upregulation and/or downregulation of motor cortical areas through the modulation of trans-callosal rivalrous interactions. Current intensities and current densities varied from 1 to 2 mA and from 0.04 mA/cm^2 to 0.63 mA/cm^2, respectively. Note that, in our review, a low number of tDCS sessions were not considered an exclusion criterion. Accordingly, we distinguished two types of study designs: those that sought a therapeutic "cumulative effect" by applying several consecutive tDCS sessions (equal or more than five sessions) and those whose design was "experimental" and delivered a few sessions (less than five sessions). The total number of delivered tDCS sessions per treatment among all included studies oscillated between 1 and up to 20 active tDCS sessions. However, from those trials seeking a cumulative therapeutic effect (16 out of 24, i.e., 66%), the large majority of them (15 out of 16, i.e., 93%) delivered ~10 sessions with a periodicity of three to five sessions per week and an average duration of 10–20 min of tDCS per session. The vast majority of selected studies were carried out on chronic stroke subjects (15 out of 24, 62%) and focused on the modulation of ipsilesional M1 (21 out of 24, 87%) resources *via* an indirect effect on spared contralesional M1 through trans-callosal inhibition. A single study (4%) investigated the effects of tDCS on the supplementary motor area (48), whereas two studies (8%) used the cerebellum as a stimulation target (53, 54). Electrode allocation was based on the 10/20 EEG system in 14 of 24 studies (58%), based on the 10/20 EEg system considering anatomical limits in 4 of 24 (16%), based on hotspot localization with TMS MEP in 4 of 24 (16%) and not reported in two studies (8%). None of them used neither MRI-based neuronavigation systems nor E-field individualized predictions. Finally, from all the selected 24 studies, only one (4%) reported having used a standard healthy MRI-based finite element (FEM) biophysical computational modeling to predict electrical current distribution and optimized *ad hoc* electrode montage accordingly (43).

Outcome measures and statistics

The current review focuses on improvements of function captured by well-established clinical tests related to gait *velocity, stability/mobility,* and *endurance* sub-functions, all contributing to gait ability which can be measured and monitored separately but is poorly informative when considered in isolation. Statistical outcomes should be interpreted cautiously given the differences between studies regarding the number of sessions, subject clinical profile, and baseline severity or the quality and availability of data. Additionally, some of the 24 studies retained for this review may have used different and non-purely clinical assessment measures than those our systematic focused on to assess tDCS impact on gait recovery which is the topic at the core of the current paper (such TMS evoked motor evoked potentials (MEP) or spatio-temporal gait parameters extracted from kinematic or electromyographic analyses). Hence, it might be the case that effect sizes calculated with such non-direct clinical measures, which we will not report on, are generally larger due to their higher sensibility in spite of being less informative for the clinically relevant gait improvements.

If one classifies the 24 selected studies considering the maximum and grand average of effect sizes across all reported tests assessing the impact of tDCS on gait and balance (between 24 and 72 h post-treatment offset) included in our selection of tasks and independently of their individual statistical significance (refer Table 1), four studies (16%) reported large effect sizes (≥0.8), six reports (25%) medium effects sizes (≥0.5), whereas, in six studies (25%), stimulation yielded low effects sizes (≤0.2). Note that *velocity* outcome measures (allowing effect size recalculation) were reported in 11 of the 24 included studies, *stability/mobility* outcomes in 10 of 24 studies, and *endurance* outcomes in only four of 24 studies. Importantly, essential data necessary to estimate effect sizes were missing for eight studies (33%) and hence could not be added to these analyses.

The four studies in which tDCS yielded large (across all different reported tasks) effect sizes involved subacute and chronic subjects who experienced significant improvement effects on mobility, gait velocity, and/or endurance, after applying either bifocal tDCS (anodal M1 ipsilesional, cathodal M1 contralesional) or anodal stimulation to the ipsilesional M1 with a cathodal supraorbital contralateral return (55–58). The number of active sessions applied in these four reports varied from 1 to 12 sessions, with intensities from 1 to 2 mA and current densities between 0.05 and 0.08 mA/cm^2. Importantly, nearly all of these tDCS studies (three out of four) carried "live" rehabilitation programs for gait and/or balance training (in regimes of multiple days), and undertook tDCS before the onset of rehabilitation sessions, hence avoiding "online" rehabilitation (i.e., performed in temporal simultaneity with tDCS treatment). Additionally, it should be noted that the pre-therapeutic "*proof of principle*" study by Tahtis and colleagues (58) even if classified among those showing high effect sizes, consisted of a single tDCS session delivered at rest without associated rehabilitation, and hence cannot be directly compared to the remaining three

TABLE 2 Item by item PEDRO scores for each individual study included in the review.

References	Item 2 randomization	Item 3 concealment	Item 4 baseline similitudes	Item 5 blinded subjects	Item 6 blinded therapists	Item 7 blinded assessors	Item 8 measures of key outcomes	Item 9 intention to treat	Item 10 reported statistical comparisons	Item 11 point and variability measures	Total/(10)
Andrade et al. (37)	1	1	1	1	0	0	1	1	1	1	8/10
Cattagni et al. (38)	1	0	1	1	1	1	1	1	1	1	9/10
Chang et al. (39)	1	0	1	1	0	1	1	0	1	1	7/10
Danzl et al. (40)	1	0	1	1	0	1	1	0	1	0	6/10
Fusco et al. (41)	1	0	1	1	0	1	0	0	1	1	6/10
Geroin et al. (42)	1	0	1	0	0	1	1	0	1	1	6/10
Kindred et al. (43)	1	0	1	1	1	0	1	1	1	1	8/10
Klomjai et al. (44)	1	0	1	1	1	0	1	1	1	1	8/10
Leon et al. (45)	1	1	1	1	0	0	1	1	1	1	8/10
Liang et al. (46)	1	1	1	1	1	1	1	1	1	1	10/10
Madhavan et al. (47)	1	1	0	1	1	1	1	0	1	1	8/10
Manji et al. (48)	1	0	1	1	0	1	1	1	1	1	8/10
Mitsutake et al. (49)	1	0	1	1	0	1	1	1	1	1	8/10
Ojardias et al. (50)	1	0	1	1	1	0	1	1	1	1	8/10
Park et al. (51)	1	0	1	1	0	0	1	0	1	1	6/10
Picelli et al. (52)	1	1	1	1	0	1	1	1	1	1	9/10
Picelli et al. (53)	1	1	1	1	0	1	1	1	1	1	9/10
Picelli et al. (54)	1	1	1	1	0	1	1	1	1	1	9/10
Prathum et al. (55)	1	1	0	1	0	1	1	1	1	1	8/10
Sacys et al. (56)	1	1	1	1	0	1	1	1	1	1	9/10
Seo et al. (57)	1	0	1	1	1	1	0	1	1	1	8/10
Tahtis et al. (58)	1	0	1	1	0	1	1	1	1	1	8/10
Utarapichat et al. (59)	1	0	0	1	0	0	1	1	1	1	6/10
van Asseldonk et al. (60)	1	0	0	1	1	0	1	0	1	1	6/10

Items description (extracted from the official web page: https://pedro.org.au/english/resources/pedro-scale/): Item 2: subjects were randomly allocated to groups, Item 3: allocation was concealed, Item 4: groups were similar at baseline regarding the most important prognostic indicators, Item 5: blinding of all subjects, Item 6: blinding of all therapists who administered the therapy, Item 7: blinding of all assessors who measured at least one key outcome, Item 8: measures of at least one key outcome were obtained from more than 85% of the subjects initially allocated to groups, Item 9: all subjects for whom outcome measures were available received the treatment or control condition as allocated or, where this was not the case, data for at least one key outcome was analyzed by "intention to treat", Item 10: the results of between-group statistical comparisons are reported for at least one key outcome, and Item 11: the study provides both point measures and measures of variability for at least one key outcome.

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studies, which delivered a regime of several days of stimulation in search of lasting effects.

The six studies yielding medium effect sizes delivered mainly anodal tDCS on the ipsilesional M1 positioning the return electrode on the supraorbital contralateral area or cathodal stimulation on the contralesional spared M1 positioning the anode in the prefrontal ipsilesional cortex (39, 42, 44, 47–49). The number of active-delivered daily sessions varied from 1 to 20 at intensities from 1 to 2 mA and current densities between 0.04 and 0.08 mA/cm^2. Importantly, four of the six studies were coupled to an online (hence simultaneous) motor rehabilitation program involving balance and/or gait training during tDCS stimulation, one study implemented a rehabilitation program immediately following each tDCS intervention, whereas one study did not couple stimulation to a planned rehabilitation program reports that tDCS sessions were followed by 1 h of motor activity.

Finally, the six studies reporting low effects applied anodal tDCS over the ipsilesional M1 leg motor area or on a scalp position lateral to Cz (according to EEG 10/20 reference system) with the cathode on a supraorbital contralateral site. The number of active sessions varied from 1 to 20 tDCS active sessions at intensities of 2 mA in all cases and current densities between 0.05 and 0.22 mA/cm^2. In three of these six studies, stimulation was combined with concomitant rehabilitation during participation in the protocol and also online rehabilitation during tDCS delivery (45, 51, 52); in one of them, online rehabilitation was combined during tDCS sessions in the absence of any associated concurrent rehabilitation program during the participation in the protocol (46). Finally, two studies applied tDCS without online concurrent rehabilitation during tDCS or any other type of rehabilitation (38, 59).

Regarding the eight studies for which we were unable to calculate effect sizes due to insufficient data in any of the outcome measures, five of them reported statistically significant improvements following active tDCS compared to sham stimulation (37, 40, 50, 53, 60); whereas, in contrast, three reports failed to find statistically significant effects of stimulation (41, 43, 54). The number of active tDCS sessions varied from 1 to 12 sessions using intensities ranging from 1.5 to 2 mA and current densities ranging between 0.05 and 0.63 mA/cm^2. The tDCS strategies used by such eight studies were either anodal stimulation over the ipsilesional M1, cathodal stimulation over the contralesional spared M1, or bifocal bi-hemispheric tDCS. For three of the eight studies, stimulation sessions were combined with online rehabilitation and while participants also followed concurrent rehabilitation programs during their participation, in one study, tDCS was immediately followed by a rehabilitation session, and one study applied tDCS along online rehabilitation but in the absence of any concurrent rehabilitation program, whereas two studies simply applied tDCS at rest without any kind of either ongoing or concurrent rehabilitation.

Finally, regarding our statistical analysis, Spearman's correlation revealed significant negative correlations between the effect size of tDCS impact on *stability/mobility* measures and the number of elapsed weeks post-stroke at treatment onset (rho $= -0.65$, $p = 0.04$). Additionally, a one-way Kruskal–Wallis test revealed statistically significant differences ($p = 0.04$) between these same outcomes at the group level. *Post-hoc* analysis revealed differences in treatments onsetting at the subacute stage (2–24 weeks) compared to the chronic stage (+24 weeks) as a group analysis ($p = 0.03$). These outcomes strongly argue in favor of an early (*subacute*) compared to a late (*chronic*) clinical therapeutic window of opportunity, with a higher potential for effective tDCS neuromodulation. Even so, given that independent variables can together affect dependent variables, we conducted standard multiple regression to better understand our results taking *stability/mobility* measures as dependent variables and treatment *intensiveness, periodicity*, tDCS current *intensity*, tDCS current *density*, and *time since stroke* as independent variables. However, no significant results were reached ($F_{(5,4)} = 0.69$, $p = 0.65$, $R^2 = 0.46$) and any of the independent variables proved to be significant predictor of *stability/mobility* improvement ($p = 0.73$, $p = 0.49$, $p = 0.87$, $p = 0.53$, $p = 0.24$, respectively).

On the other hand, a Kruskal–Wallis test reached significance ($p = 0.03$) between the effect size of tDCS on *stability/mobility* and stimulation strategy arguing in favor of the bifocal stimulation montage (anodal M1 ipsilesional and cathodal M1 contralesional) with respect to anodal M1 ipsilesional stimulation alone with supraorbital contralesional cathodal return. No other statistically significant correlations or group differences were found between effect sizes in *velocity* or *endurance* vs. treatment *intensiveness, periodicity*, tDCS current *intensity, stimulation strategy*, tDCS current *density*, and *time since stroke* episode, or between effect sizes in *stability/mobility* vs. treatment *intensiveness, periodicity*, tDCS current *intensity*, or tDCS current *density*. Data distribution of the two measures showing significant group differences is represented in Figure 2.

Discussion

The current systematic review summarizes and evaluates peer-reviewed studies published in English language journals until 7 February 2022, assessing the use of tDCS for gait rehabilitation in stroke subjects. We aimed to characterize current state-of-the-art and assess the efficacy of most common strategies and methodologies to improve future therapeutic applications in stroke subjects for gait and associated motor and cognitive deficits. Our systematic review also implemented a complementary statistical strategy to explore the influence

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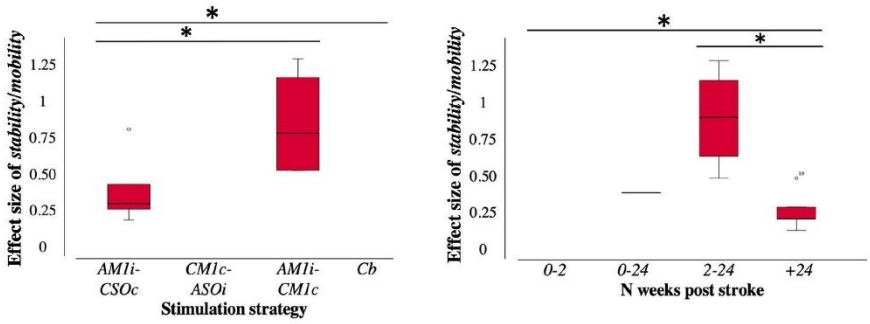


FIGURE 2
Distribution of effect sizes according to the characteristics of the retained studies ($n = 24$ publications included). Study characteristics were grouped and categorized [time since stroke episode (*N weeks post-stroke*, categorized in 4 groups: from 0 to 2 weeks, from 0 to 24 weeks, from 2 to 24 weeks, + 24 weeks) and *stimulation strategy* (categorized in 4 groups: anodal ipsilesional M1 or SMA or leg area tDCS and contralateral supraorbital or prefrontal or inion return, cathodal contralesional M1 tDCS and ipsilesional supraorbital return, dual anodal and cathodal M1 bilateral stimulation, cerebellar cathodal stimulation)]. Only measures that showed statistically significant differences in the Kruskal Wallis test are presented in the figure form stability/mobility effect size with respect to time since stroke (N weeks post-stroke) and stimulation strategy (electrode positions). Boxplots were drawn from the 25th to the 75th percentile and the horizontal line indicates the mean. Boxplots were generated in SPSS (IBM, USA). Effect size representation in boxplots was normalized from 0 = minimum, to 1.3 = maximum. AM1i, anodal primary motor cortex ipsilesional; ASOi, anodal supraorbital ipsilesional; Cb, cerebellum; CM1c, cathodal primary motor cortex contralesional; CSOc, cathodal supraorbital contralesional. *Statistically significant, $p < 0.05$.

of time post-stroke at treatment onset, tDCS current intensity and density, tDCS treatment intensiveness, tDCS treatment periodicity, and tDCS strategy on effect sizes achieved by individual studies on tasks assessing gait *velocity*, *endurance*, and *stability/mobility*. The influence of these specific parameters was explored to shed further light on how they might impact modulatory ability so that the most efficient stimulation parameters and strategies could be planned to maximize post-injury plastic adaptive reorganization across specific windows of opportunity and subject's conditions.

Our review work identified at least 10 studies (out of 16 items with available data, and a total set of 24) with moderate or high effect sizes suggesting that tDCS remains a promising strategy to facilitate the recovery of different aspects of gait following stroke. Nonetheless, according to prior meta-analyses on this topic, our own analyses on general effect sizes revealed a rather modest impact at the group level (28–32). On such a basis, we emphasize the role of inter-individual differences in response to treatment (in turn determined by factors such as baseline clinical severity and time between stroke event and treatment onset or stimulation strategy and interactions thereof) as responsible for weakening therapeutic impact in large subject cohorts. In this context, we hypothesize that therapeutic individualization might be one of the key strategies to improve the clinical success of tDCS on gait rehabilitation following stroke. This effort first needs to be articulated by designing clinical trials which stratify subjects on the basis of baseline symptom severity at the time of treatment onset and "tailored" neuromodulation strategies based on pre-defined biomarkers predictive of beneficial responses to tDCS treatment such as (among additional potential others): corticospinal tract integrity (64–68), lesion location, extent, time post-injury and state of ongoing activity on lesional, perilesional, and spared associated areas (18, 69–73). Unfortunately, to date, very few of these relevant variables have been systematically tested in clinical trials and their influence had been explored in multivariate studies combining them rather than addressing each one at a time.

Neurostimulation strategies for gait rehabilitation

Our detailed inspection of available evidence for the effects of tDCS on gait rehabilitation following stroke according to well-stated criteria (a total of 24 publications included in the analyses of the current review) demonstrates that (as is the case for upper-hand rehabilitation peer-reviewed research) a large majority of therapeutic tDCS stroke studies in this field rely on the manipulation of trans-callosal interhemispheric inhibitory interactions (17, 74) (Figure 3A). Furthermore, our own statistical analyses suggest that immediate post-treatment effect sizes across individual studies and outcome *stability/mobility* measures scaled significantly (rho = −0.65, $p = 0.04$) with

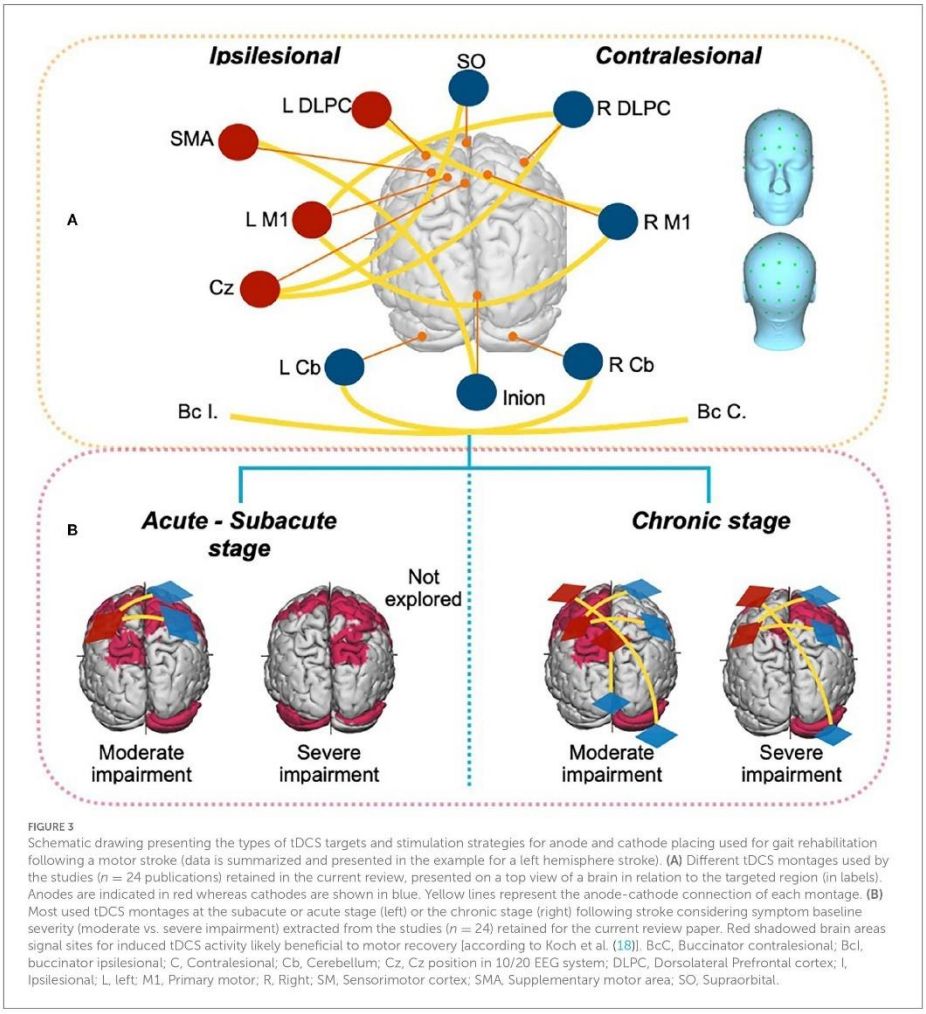


FIGURE 3
Schematic drawing presenting the types of tDCS targets and stimulation strategies for anode and cathode placing used for gait rehabilitation following a motor stroke (data is summarized and presented in the example for a left hemisphere stroke). **(A)** Different tDCS montages used by the studies ($n = 24$ publications) retained in the current review, presented on a top view of a brain in relation to the targeted region (in labels). Anodes are indicated in red whereas cathodes are shown in blue. Yellow lines represent the anode-cathode connection of each montage. **(B)** Most used tDCS montages at the subacute or acute stage (left) or the chronic stage (right) following stroke considering symptom baseline severity (moderate vs. severe impairment) extracted from the studies ($n = 24$) retained for the current review paper. Red shadowed brain areas signal sites for induced tDCS activity likely beneficial to motor recovery [according to Koch et al. (18)]. BcC, Buccinator contralesional; BcI, buccinator ipsilesional; C, Contralesional; Cb, Cerebellum; Cz, Cz position in 10/20 EEG system; DLPC, Dorsolateral Prefrontal cortex; I, Ipsilesional; L, left; M1, Primary motor; R, Right; SM, Sensorimotor cortex; SMA, Supplementary motor area; SO, Supraorbital.

time post-stroke, and show that the implementation of tDCS rehabilitation programs during the subacute phase achieved higher effect sizes than those acting at later stages. More specifically, interventions onsetting up to 2–24 weeks post-stroke (sub-acute period), when perilesional and spared brain systems are more prone to functional reorganization, may have more chances to achieve clinical success than those operating at a later chronic stage.

The current systematic review and associated analyses also suggest that tDCS might be more prone to increase the activity of spared residual perilesional networks around the areas of stroke damage, hence better suited than the manipulation of spared/intact remote circuitry (with similar input-output connectivity patterns and neurophysiological coding strategies for the impaired functions) to effectively support the remapping of lost functions. Concomitantly, contralesional hyperactivity

generated by a partial loss of the inhibitory drive from the injured hemisphere may play a compensatory role, acting as a recovery-promoting mechanism, especially at acute stages following large lesions (18, 75). In this regard, the inhibition of perilesional activity mediated by a local over-expression of extra synaptic $GABA_A$ receptors in neuronal resources hosted in the penumbra periolesional region around the stroke area and beyond has been reported as persisting for more than a month. Most importantly, reversing this inhibition has been shown to improve motor prognosis (76, 77). The latter observation supports the notion that the modulation of such activity from either local (i.e., by enhancing perilesional activity directly on the stroke region) or distant (i.e., by suppressing areas from the spared contralateral hemisphere exerting an abnormally intense inhibitory drive onto perilesional regions) targets is key to achieving some level of functional remapping and benefit clinical recovery.

Similar "*push and pull*" rivalrous interaction mechanisms mediated by mutually inhibitory projections have also been reported between nodes of widespread cerebral and cerebellar motor networks. In this regard, during the transition from the acute to the subacute post-stroke phase, decreases in ipsilesional M1 function contrasting with lesion size and location-dependent activity increases of ipsilesional premotor regions, supplementary motor area (SMA), the contralesional cerebellum, and the contralesional M1 have been reported and interpreted as a spontaneous brain compensatory reactions able to preserve motor output (75, 78, 79). On such basis, most studies focusing on post-stroke gait rehabilitation in the sub-acute phase have opted for strategies inhibiting contralesional M1 activity and/or by exciting ipsilesional M1 systems, yielding significant improvements at the group level. Nonetheless, such effects have been proven to be quite heterogeneous across subjects and studies. Moreover, a lack of clinically relevant effects has also been often reported for net excitatory effects of stimulation delivered to the ipsilesional hemisphere.

In such context, the notion of a bell's shape function relating post-stroke clinical severity at baseline and levels of functional recovery, emphasizing the importance of a specific window of gait impairment that maximizes a beneficial impact of stimulation has emerged in the domain. Exactly to this regard, recent connectivity-based clinical predictive models exploring the influence of damaged white matter projections in stroke subjects (18), based on fractional anisotropy measures, suggest that subjects with mild-to-intermediate baseline impairments showing greater levels of sparing in relevant bilateral structural connectivity bundles [involving not only in primary motor (M1), ventral premotor (PMv) but also the ipsilesional inferior frontal gyrus (IFG), and bilateral parietal cortices, somatosensory areas and attentional regions] tended to achieve greater recovery levels than those in which such connectivity was damaged (Figure 3B.1). Nonetheless, 3-month post-injury, relevant structural connectivity of moderately injured subjects associated with significant spontaneous recovery changes, particularly when subcortical structures of the injured hemisphere were affected, encompassed the contralesional cerebellum, and of frontal, premotor, and somatosensory areas. Three months after stroke, spontaneous plasticity processes have been shown to weaken, and at such an advanced stage, sustained contralesional hyperactivity is associated with worse clinical outcomes, hence considered deleterious for the recovery of mild motor impairment, whereas paradoxically, it might remain beneficial for large corticospinal tract lesions (73, 78–80). This same predictive model reveals that for subjects with mild-to-intermediate motor impairments, relevant structural connectivity warranting recovery varies with respect to severely affected subjects, highlighting the need for individualized interventions (18).

All in all, severity- and phase-dependent vicariation (81) and neural compensation by functional remapping or reorganization in novel pathways and cortical and subcortical areas (including the cerebellum) underline the chances for adaptive plasticity and clinical recovery of motor function (75) (Figure 3B.2). Most importantly, the dynamic nature of such phenomenon can be captured by computational models, which might anticipate prognosis and inspire more effective subject-customized therapies to regain motor abilities. However, current connectivity-based predictive models for motor recovery have been developed for upper limb impairments, hence set up rules might not necessarily directly apply and adequately inform gait rehabilitation, highlighting the need to extend such work (18) to different motor, sensory, and cognitive domains.

Identifying optimal strategies to increase TDCS efficacy in post-stroke gait rehabilitation

Our analyses revealed statistically significant correlations ($p = 0.03$) between the effect size of tDCS treatment on *stability/mobility* and stimulation strategy, arguing in favor of the bifocal stimulation montage (anodal M1 ipsilesional and cathodal M1 contralesional) with respect to anodal M1 ipsilesional stimulation alone with supraorbital contralesional cathodal return. This suggests that tDCS studies implementing strategies coherent with current knowledge or state-of-the-art connectivity-based predictive models, sensitive to stroke phase-specific activity changes ultimately tied to spontaneous adaptive plasticity may have the potential to result in greater clinical effects than those that did not. This outcome highlights the importance of closely monitoring and characterizing (from the acute to the chronic post-stroke phase) brain activity dynamics related to adaptive spontaneous plasticity experienced by subjects and using stimulation to guide and promote that in order to optimally enhance recovery.

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Additionally, technical aspects related to tDCS delivery such as the electrode placement, the electrode montages, or the magnitude of delivered currents but most importantly predicted current density (V/m) on the pursued cortical target have been found to influence therapeutic outputs. Whereas, in contrast, no significant correlations were found between post-tDCS effect sizes and delivered current intensity (in mA) or density (intensity/electrode surface). Further attempts to identify potential relationships between the effect sizes of tDCS impact and the latter factor failed due to the lack of available data to accurately estimate such parameters in a significant number of studies. Nonetheless, one main and single reason explains this outcome; delivered field intensity (in mA) which was indeed well reported in a majority of studies or electrode current density (in mA/cm^2) inform poorly on peak current density (V/m) achieved at a given cortical target. In the absence of direct intracranial recordings, an accurate estimation of electric field strength ($|E|$) or the normal component of the electric field (nE) at target would require the computation of a head and brain biophysical model simulating current distribution based on individual subject MRI-based models (at worst, on a standard representative MRI volume) which a great majority of studies lacked (34, 82, 83). To overcome this limitation in the future, studies should be encouraged to include model-based estimations of the predicted electrical field (specially $|E|$ and |nE|) revealed by standard or individualized biophysical head–brain FEM models.

Most current tDCS multichannel systems (i.e., using more than two electrodes) allow for the simultaneous stimulation of different cortical areas. Moreover, the uses of such technologies guided by MRI-based computational biophysical modeling systems such as NeMo-TMS (84) ROAST (85), or SimNIBS (86) can contribute to a more precise control of the distribution of electrical flow, providing a tool to fulfill subject-customized optimized stimulation strategies. Unreasonably, despite biophysical FEM computational models are being used in other clinical settings is yet to become mainstream to be used in post-stroke motor rehabilitation, to adequately dose tDCS and assist the design of individually tailored interventions. Regardless, precise and reliable targeting informed by current distribution models taking into account individual anatomical head/brain models is not the only variable that must be mastered to adequately predict outcomes. For example, given that stroke is indeed a network dysfunction, the status of time-correlated oscillatory activity and interregional synchrony operating at different frequency bands seems also paramount in this regard. More specifically, decreases in posterior alpha (10–12 Hz) and beta-band oscillation power (14–20 Hz, closer to the lesion), global increases of delta (1–4 Hz) and theta (4–8 Hz) synchronization, broadband interhemispheric oscillatory asymmetries (15–50 Hz) with lower power in the injured hemisphere and alpha and beta power decreases related to changes of functional connectivity (87–89) are some post-stroke electrophysiological features considered for optimizing therapeutical interventions at the group or the individual level. Once such individual biomarkers are tested and validated, two tES modalities, Transcranial Alternate Current Stimulation (tACS) and Random Noise Stimulation (tRNS), are able to entrain and desynchronize, respectively, beta- and gamma-related activity in motor networks, open the possibility to design novel stimulation strategies which might show higher efficacy by manipulating the synchrony and correcting the abnormal rhythms, rather than simply operating on the activity levels.

Setting the stage for future innovative TDCS strategies in the rehabilitation of stroke

Promising tDCS stimulation strategies and predictive models based on spurring beneficial (i.e., adaptive) dynamic changes or limiting maladaptive reorganization following stroke for rehabilitation are continuously being proposed. Given current knowledge on the importance of involved structural connectivity for motor rehabilitation depending on lesional stage and damage severity, plus the added possibility of using multichannel (hence multipolar or multi-site) tDCS systems combined with new electric flow distribution prediction algorithms with higher focality, two innovative approaches that to date have never been systematically developed are called to gain momentum; however, our propositions still need to be clearly discussed and explored in controlled trials to assess their feasibility. First, as proposed by Otal et al., a multi-site stimulation strategy aiming to boost the re-learning and consolidation of motor skills could be achieved by driving simultaneous activity increases of ipsilesional sensorimotor cortex S1, ipsilesional M1, and the anterior lobe of the contralesional cerebellum, while simultaneously decreasing contralesional M1 contributions (Figure 4). In the same vein, a viable alternative adapted to even more severe subjects may consist of upregulating the activity of the ipsilesional M1 while downregulating activity in contralesional primary sensorimotor areas S1, contralesional M1, and contralesional anterior cerebellum (90).

Second, some studies have recently shown that motor and attentional systems [particularly the dorsal attentional network (DAN)] interact during stroke rehabilitation and influence chances for recovery (6, 13, 14). On such basis, a multi-site and multi-domain approach (15, 91) aiming to simultaneously increase activity in the dorso-lateral prefrontal cortex (DLPC) and the cerebellum in moderate-to-severely affected subjects in subacute or chronic stroke stages (refer Figure 5) could potentiate the therapeutic effect of tDCS on post-stroke gait impairments by acting in a synergistic manner on two separate

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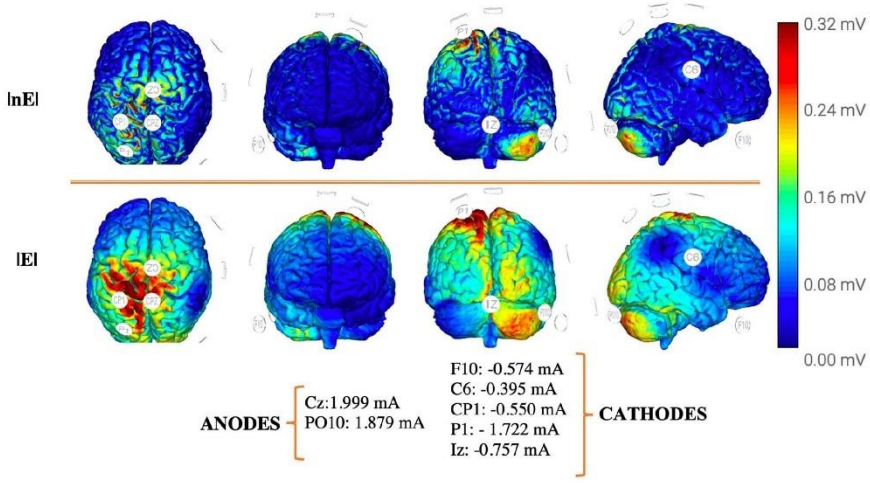


FIGURE 4
Proposal for an optimized multipolar stimulation solution for 'gait/motor rehabilitation' [see Otal et al. (90)] following a unilateral stroke motor lesion affecting the left hemisphere, based on a standard 8 channels tDCS device, electrode size: πcm^2 Ag/AgCl, aiming at delivering 0.25 V/m at each target [(1) M1 ipsilesional, (2) SM cortex ipsilesional, and (3) anterior cerebellum contralesional] on the basis of 4mA total injected current, and a constrained by a maximum of 2 mA on each individual electrode. Model optimization has been generated from a *headreco* routine (FEM) of the MNI152 template in SimNIBS 3.2.3 maximizing focality while controlling the E-field strength. Pictures were extracted from the Neuroelectrics Instrument Controller® interface. Cb, cerebellum; DLPC, dorsolateral prefrontal cortex; M1, primary motor; SM, sensorimotor cortex; V/m, volts per meter; E, total field strength; nE, normal component of the electric field.

but associated cognitive domains subtended by two different networks. Despite the potential of the cerebellum to contribute to motor relearning, key challenges still need to be addressed before it can be used as a clinical target. Especially, given the particular anatomy of the cerebellum and the dependency of electrophysiological effects on the spatial distribution of neurons and electric field distribution, different cell populations in the cerebellar cortex can antagonistically respond to transcutaneous stimulation. Therefore, it is necessary to better understand the natural effect of cerebellar stimulation and identify individual online responses adequately to dose stimulation for clinical applications (92–95). Additionally, another key element to optimize the therapeutic impact of tDCS relies on the design of stimulation strategies that consider state-dependency principles, integrating the level and nature of the activity operating on the targeted region (i.e., monitoring brain activity through EEG or fNIRS approaches) and its associated networks at the time of stimulation. As mentioned above, the distributed nature of stroke, involving alterations of local and network excitability and metabolism (including dysfunction in rhythmic coding and local and interregional synchronization events), will require the development of multi-site stimulation technologies that are able to operate on different sets of extended networks (instead of on just a few isolated nodes), allowing the simultaneous excitation, inhibition, synchronization, or desynchronization of local or distant nodes belonging to areas of the same or different networks.

Overall, neurostimulation will be in a better position to make significant contributions to clinical practice in the field of gait recovery (as for any other cognitive, motor, or sensory impairment) by: (1) improving our understanding on the anatomical systems and neurophysiological mechanisms facilitating spontaneous recovery mechanisms of such functions; (2) considering individualized stimulation protocols based, among other relevant factors, on baseline clinical severity, lesion location and extent, clinical phase and after ascertaining residual activity and preserved functional connectivity; (3) through the development of individual MRI-based computational modeling of electrical current distribution to optimize and customize interventions according to simulations, which take into account each subject's head and brain anatomical features; (4) considering the combination of neurostimulation with

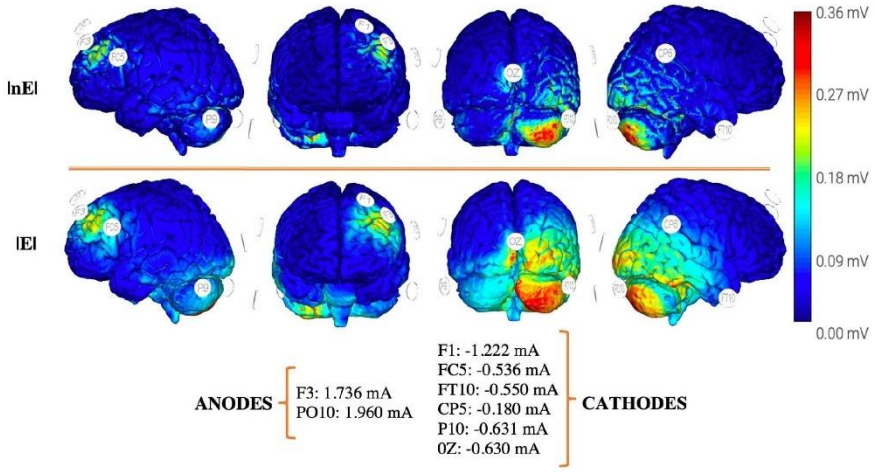


FIGURE 5
Proposal for an optimized multipolar stimulation strategy implementing "combined cognitive/motor rehabilitation" [see Wessel and Hummel (91)] following a unilateral stroke motor lesion in the example affecting the left hemisphere, based on a standard 8 channels tDCS device, electrode size: πcm^2 Ag/AgCl, aiming at delivering 0.25V/m at each target [(1)DLPC ipsilesional and (2) anterior cerebellum contralesional] on the basis of 4mA total injected current, and a constrained by a maximum of 2 mA on each individual electrode. Model optimization has been generated from a *headreco* routine (FEM) of the MNI152 template in SimNIBS 3.2.3 maximizing focality while controlling the E-field strength. Pictures were extracted from the Neuroelectrics Instrument Controller® interface. Cb, cerebellum; DLPC, Dorsolateral Prefrontal cortex; V/m, volts per meter; E, total field strength; nE, normal component of the electric field.

robust rehabilitation programs and other assistive tools (e.g., robot-assisted gait training, peripheric stimulation, trans-spinal stimulation) with priming, synergistic, or facilitatory effects, either during the *online* delivery of the stimulation and/or concurrently with neuromodulation sessions; (5) integrating real-time *online* monitoring tools of cortical activity during stimulation (either by EEG, fNIRS, or behavioral outcomes) allowing flexible and individualized treatments adapted to changes of cortical state eventually using stimulation as a part of embedded real-time close-loop technologies; Finally, (6) by implementing novel stimulation strategies able to modulate activity simultaneously different brain regions or networks and operate simultaneously on different motor, sensory or cognitive domains all contributing to regain adaptive gait.

Methodological limitations

A number of limitations affecting our review paper should be mentioned and called for caution when interpreting our results: (1) We only included randomized clinical trials published in English; hence, we might have overlooked relevant studies published in other languages that may have nuanced the outcomes of our review work and associated statistical analyses; (2) We applied astringent selection criteria hence included only high-quality controlled studies. Accordingly, we left aside a number of "pilot" studies which could have eventually provided interesting information on the topic at hand and further tuned some of our findings; (3) Data included in our study came from reports implementing a diversity of designs, not always focusing on the same outcome measures nor assessing at such identical follow-up points, a fact that could have also contributed to weaken the significance of some of the reported outcomes; (4) To minimize the impact of scarcity of data or missing values for some studies, we selected our measures of choice for tDCS impact and the time points at which those were tested among those most frequently represented in our sample; consequently, our conclusions apply to such specific conditions and we cannot rule out if they may vary whether other measures or time points were to be considered; and (5) To classify the selected studies according to their effect sizes and to later explore the influence of several variables on tDCS outcomes, we focused on the highest effect size of all

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available tasks (among those selected as reference tasks in the review) and we estimated a grand average of effect sizes across multiple available tasks. Although effects sizes were all calculated on a common basis, the final estimates might not be equally representative of gait changes induced by tDCS and may vary across studies.

Summary and conclusions

We here analyzed high-quality studies assessing the impact of tDCS on post-stroke gait (by means of mobility, endurance, and velocity measures), and quantitatively assessed the impact of delivered tDCS intensiveness, tDCS treatment periodicity, post-stroke time at tDCS treatment onset, tDCS stimulation strategy, tDCS current intensity, and density for available gait selected measures, assessed 24–72 h after treatment offset. Statistically significant negative correlations and differences were found between the duration of the post-stroke period at the time of treatment onset and effect sizes induced by tDCS on gait *stability/mobility* outcome measures, suggesting that early treatments at the subacute stage achieve better outcomes than those applied at the chronic stroke phase. Moreover, statistically significant differences were found between *stability/mobility* and the stimulation strategy (electrode placement) arguing in favor of bifocal or dual tDCS montages (anodal M1 ipsilesional combined with cathodal M1 contralesional).

Transcranial DCS has shown promise as a co-adjuvant therapeutic approach in the rehabilitation of motor and cognitive deficits following stroke. It has been thoroughly tested and studied in the recovery of post-stroke upper limb recovery, for which a recent structural connectivity-based predictive model of areas guiding potential recovery exists. The current review extends this analysis to the field of post-stroke gait deficits and provides a basis to foster further work in this area. Our study emphasizes the need to develop and test new stimulation rationals which focus on the modulation of new sets of relevant targets organized in extended networks and consider the simultaneous modulation of systems subtending different motor, sensory, or cognitive domains with a common bearing on gait function. Collaterally, our study also contributes to an ongoing debate about the consequences of assessing tDCS outcomes by estimating group averages from cohorts of subjects receiving the exact same treatment regardless of their distinctive anatomical, neurophysiological, or clinical features. The pros and cons for tDCS clinical trials to apply individual subject-customized strategies factoring in stroke severity, time course, stroke type (ischemic vs. hemorrhagic), lesion site combined with the integration of anatomically- and biophysically based electrical field computational simulations remain an ongoing discussion.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XC-T planned and performed the literature search, analyzed the data, and drafted the first version of the manuscript. AV-C and MC conceptualized and coordinated the study, verified the selection of retained literature and set up a strategy for data analysis, data presentation, and interpretation. AV-C and MC contributed with XC-T to write the final manuscript after multiple iterations. MF and RM provided expertise on several stages of the project on the literature search and data interpretation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation to improve gait in post-stroke patients
Xavier Corominas Teruel

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4.1.2 Manuscript 2: EPICURUS: E-field-based spatial filtering procedure for an accurate estimation of local EEG activity evoked by Transcranial Magnetic Stimulation

EPICURUS: E-field-based spatial filtering procedure for an accurate estimation of local EEG activity evoked by Transcranial Magnetic Stimulation

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ABSTRACT

Background: The concurrent use of Transcranial magnetic stimulation (TMS) with electroencephalography (EEG) is increasingly integrated in research and clinical protocols to provide proof of effect by magnetic pulses. However, a reliable identification of evoked local EEG activity over TMS targeted cortical sites remains still challenging. **Methods**: Here we present EPICURUS, a novel EEG spatial filtering approach, by which, individual MRI based simulations of TMS- electrical fields (E-fields) guide the reconstruction of TMS evoked o EEG signals originated in the primary motor cortex, minimizing crosstalk from non relevant more distant sources. **Results**: A reduction of late Transcranial Evoked-Potentials (TEPs) components (>100 ms post pulse onset) suggest our E-field-based spatial filter approach efficiently reduced intrusion of non-locally relevant distant sources engaged by TMS, particularly when combined with a suppression of auditory entries. **Conclusion**: The individually customized E-field-based spatial filtering procedure here developed for TMS-EEG datasets shows promise improving the spatio-temporal mapping of primary sources activated by magnetic pulses.

Keywords: Brain Stimulation, Electrical field simulation, Transcranial magnetic stimulation, concurrent TMS-EEG, spatial filtering.

HIGHLIGHTS

1. Concurrent TMS-EEG recordings are a well-established tool to monitor brain state of activity and provide proof of effect and target engagement.
2. The lack of spatial specificity of EEG prevents an accurate estimation of the locally activated EEG sources, hindering the interpretation of TMS effects.
3. We here developed a spatial filtering approach for TMS-EEG based on individual estimation of E-field spread following stimulation.
4. Our E-field spatial filter combined with auditory constraints reduced non-relevant distant signal components and improved the reliability of local EEG estimates.

INTRODUCTION

Electroencephalography (EEG) is a neurophysiological approach increasingly employed as the optimal companion to monitor the impact of Transcranial Magnetic Stimulation (TMS) on brain activity. However, the activity recorded by such a high-temporal resolution technique after a TMS pulse remains spatially unspecific, and reflects a combination of mixed cerebral sources directly or indirectly activated by TMS. This phenomena occurs because besides relevant local direct effects on the targeted cortical site TMS engages activity in interconnected distant areas and networks [1], while also increasing function in non relevant regions driven by TMS-generated sensory input in the auditory, tactile and vestibular domains [2,3].

Here, we present an innovative approach to isolate activity genuinely elicited locally by TMS pulses within the modeled boundaries of a targeted cortical region, while minimizing unspecific contributions from non-relevant distant sources. The method dubbed EPICURUS (**E**-field based s**P**atial f**I**ltering for **C**oncurrent EEG-TMS data **U**sed to ta**R**get so**U**rce **S**ignal) has been designed as an spatial filter for TMS-EEG datasets, and combines E-field simulations [4] with a Minimum Norm-like EEG source Estimator (MNE), minimizing signal leakage and crosstalk from non-stimulated brain locations [5-7].

MATERIALS AND METHODS

Participants

To test EPICURUS performance, we used it to analyze a TMS-EEG dataset of n=18 healthy participants (10 females, 8 males, 26±5 years old), all right-handed, meeting safety criteria for MRI [8] and TMS [9], who provided written consent to participate in the study. Our protocol followed the Declaration of Helsinki and received approval by a local ethical committee (*Comité de Protection des Personnes, Ile de France I*).

TMS-EEG protocol and data processing

Participants received 80 single TMS pulses (with a variable interpulse interval of 5-7sec) over the left primary motor cortex (M1) while we continuously recorded (25kHz sampling rate) scalp EEG activity with a 65 electrode amplifier (ActiChamp, Brain Products) and active electrodes placed following the International 10/10 system coordinates. The left M1 'hotspot' was defined as the optimal cortical location for eliciting at rest Motor-Evoked Potentials in the First Dorsal Interosseous (FDI) muscle of the right hand [10]. TMS was administered with a D70 mm figure-of-eight coil attached to a biphasic stimulator (Magstim Rapid2) at a fixed intensity of (60% of the maximal stimulator output; MSO). On each participant, TMS coil position was maintained consistently throughout the experiment using an MRI-frameless stereotactic system (Brainsight, Rogue Solutions Inc., Montréal, QC, Canada).

The TMS-EEG dataset was processed using the MATLAB-based toolbox EEGLAB [11] and its TMS–EEG signal analyzer plugin (TESA [12]). Data was epoched around the TMS pulse (from -1200 to 1200ms – w.r.t. – the TMS pulse) and baseline corrected (from -500 to -10ms). Artifacted channels were temporarily removed from analyses. TMS-pulse electromagnetic (from -2ms to 12ms w.r.t the TMS pulse) and recharging artifacts (visually identified [13]) were removed and interpolated using a spline function. Data was downsampled to 1kHz and decomposed into independent components (ICA) to remove ocular artifacts. Next, the SOUND algorithm was used for noise suppression, replacement of removed channels, and signal re-referencing to a common average [14]. The SSP-SIR algorithm was then used to suppress TMS-evoked muscle artifacts [15] and a second ICA was computed to remove residual continuous muscle artifacts. Finally, data was bandpass filtered with a fourth-order Butterworth filter (2-45Hz) and baseline corrected.

E-field-based spatial filter

Our pipeline integrates a previously developed spatial filter approach based on cross-talk functions (DeFleCT; [5]) with E-field distribution modeled using SimNIBS 4.0 [4]. First, we reconstructed individual finite element head models (FEM [16]) for each participant and

computed the leadfield matrix employing the Helsinki BEM framework [6] from the FEM model. We simulated the E-field distribution induced by TMS at the intensity of 60% MSO with SimNIBS (version 4.0, 114.7 dI/dtmax=100% MSO; [17]), using TMS coil positions recorded during the experimental session. We employed the spatial distribution of each participant's E-field to identify the cortical area most impacted by TMS (>80% E-field peak strength; region of interest–ROI). Next, a spatial filter with distributed source minimization constraints (i.e., crosstalk from outside the established ROI) was built for each participant. The spatial filtering strategy mirrors Special Case 2 of the DeFleCT implementation [5], using an L2 minimum-norm estimate with a normalization constraint tailored to the TMS targeted sources combined with a noise minimization constraint (See Figure1). To validate our approach and explore the capabilities of the filter, we implemented an additional filtering procedure; a discrete source constraint minimizing signal components arising from sources in the auditory cortex (~Brodman areas 22,41 and 42; [18]; bilaterally delineated on each the individual head models). Hence, the original EEG signals were filtered to generate two dataset conditions: one using the E-field-based filter alone and another adding to the former the above-mentioned auditory constraints.

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Spatial filter validation and statistical analysis

To validate our spatial filtering pipeline EPICURUS, we first identified TEPs from all available EEG sensors (Figure 2.A.1, A.2) and characterized their latency, morphology and topographies. Next, we analyzed TEPs from a cluster of electrodes in the vicinity of the TMS stimulation site (i.e., mean signal from C1, C3, CP1 and CP3; sensor-level original signal; Figure 2.A.3). Additionally, we employed a classical MNE estimator, without noise minimization (i.e., source-level original signal), to reconstruct EEG time-series directly from the sources impacted by the stimulation (i.e., the sources identified within the E-field-simulation-based ROI; (Figure 2.A.3)).

The sensor-level and source-level original TEPs served as *reference data* conditions for our analysis, and were statistically compared to the spatially filtered signals (E-field-based and E-field-based with auditory constraints). Comparisons were performed with non-parametric cluster-based permutation statistics employing one-way 1x3 ANOVA (FieldTrip; [19,20]; 50000 iterations, $\alpha = 0.05$, $p < 0.05$, time-window: from 0ms to 400ms -w.r.t. TMS pulse). Whenever needed, post-hoc tests were conducted using cluster-based non-parametric statistics with two-tailed dependent-samples t-tests (50000 iterations, $\alpha = 0.05$, $p < 0.05$), employing Bonferroni correction for multiple comparisons.

RESULTS

Comparisons between E-field-based filtered datasets and the original source-level and sensor-level signals revealed significant differences across, identifying a significant cluster ~175ms following TMS pulse onset ($F(2, 32) = 9.1$, $p = 0.002$; Figure 2.B.1). Post-hoc comparisons indicated significant differences between the source-level and the sensor-level original signals (paired t-test: $t = 3.6$, $p_{corrected} = 0.006$), and between the E-field-based filtered signal and sensor-level original signal ($t = 2.9$, $p_{corrected} = 0.04$). However, no significant differences were observed between the E-field-based filtered signal and the original source-level dataset ($t = 2.3$, $p_{corrected} = 0.3$).

Comparisons between E-field-based filtered signal with auditory constraints and the original source-level, and sensor-level signals revealed significant differences across signals, and revealed two clusters with different latencies (cluster 1, ~115ms, $F(2, 32) = 7.7$, $p = 0.03$; cluster 2, ~175ms, $F(2, 32) = 9.7$, $p = 0.001$; Figure 2.B.2). Post-hoc comparisons indicated significant differences between the E-field-based filtered with auditory constraints and the sensor level original signal on both clusters (cluster 1, ~115ms, $t = -4$, $p_{corrected} = 0.01$ / cluster 2, ~175ms, $t = 3.2$, $p_{corrected} = 0.01$), and between the E-field-based filtered with auditory constraints and the source level original signal on the first cluster (cluster 1, ~115ms, $t = -2.7$, $p_{corrected} = 0.05$).

Finally, the comparison between the E-field-based filtered signal and the E-field-based with auditory constraints conditions revealed significant differences in the same two late latency clusters described in the previous analysis (cluster 1, ~115ms, $t = -4.5$, $p_{corrected} = 0.0004$; cluster 2, ~175ms, $t = 4.6$, $p_{corrected} = 0.001$; Figure 2.B.3).

DISCUSSION

Here we designed and implemented EPICURUS, a spatial filtering approach for TMS-EEG data that implements the TMS coil position during the experimental session, the E-field distribution biophysical model, and MNE source reconstruction methods minimizing signal leakage and crosstalk from distant brain sources.

Our results revealed three key findings. First, the shape and latency of E-field-based filtered TEPs (with and without auditory constraints) were comparable to those originally recorded or estimated (source and sensor level), supporting the reliability and robustness of an approach that does not alter the overall time course of TMS evoked signals. Second, E-field-based filtered TEPs (with and without auditory constraints) showed lower amplitude than the sensor-level original TEPs, especially at later components (> 100 ms post TMS pulse onset). However, only TEPs processed with EPICURUS adding auditory constraints reached statistically significant differences compared to original source-level TEPs. Third, signals reconstructed with the E-field-based filtering method differed from those reconstructed with E-field-based filtering combined with auditory constraints, particularly ~115ms.

Prior work has shown that late TEP components, such as those present in our data at ~115 and ~175 ms (N115 and P175) with fronto-central topographies (Figure 2.A.2), typically reflect the engagement of distant brain regions directly or indirectly activated by the original TMS pulse [1,2,21-23]. We here hypothesize that statistical differences between filtered and original signals here reported may arise from the attenuation of distant sources which, at least in part, contribute to indirect multisensory EEG responses to TMS. A likely direct link to auditory evoked activity is supported by our finding showing that the addition to EPICURUS of auditory constraints significantly reduces the amplitudes of such late TEP components.

Although our filtering approach efficiently suppressed the contribution of TMS activity associated to multisensory responses, the primary aim of EPICURUS is to increase the reliability of local evoked responses minimizing the distant non-relevant sources. The suppression of multisensory responses in our TMS-EEG example is an indirect consequence of this aim. To effectively suppress multisensory responses, proper experimental procedures during experimental sessions (e.g., noise masking, control conditions; [21]) potentially combined with other analysis strategies, will be necessary [24–26].

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AND CODE AVAILABILITY

The datasets produced and/or analyzed during the current study cannot be made publicly available due to institutional regulations. However, they can be obtained from the corresponding and/or first authors of the manuscript upon reasonable request. MATLAB codes for the pre-processing and filtering implementation can be found at the permanent link: *https://github.com/XavierCorominas/Source-Based-filters-for-brain-stimulation-readouts*.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Conceptualization: XC-T, TM, MB, AV-C. Methodology: XC-T, TM, MB. Data curation and analysis: XC-T. Funding acquisition: CG, MB, AV-C. Supervision: MB, AV-C. Writing – original draft: XC-T, AV-C & MB. Writing – review and editing: XC-T, TM, CL, CG, MT-C, MB, AV-C.

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FIGURES

Figure 1. Methodological overview. Illustrative step-by-step procedure of the E-field-based spatial filtering pipeline EPICURUS. Raw TMS-EEG data (1) was preprocessed and cleaned to remove artifacts from each of the n=18 participant datasets (2). In parallel, MRI-based head models and E-field distributions on each MRI volume were computed for each participant. Finally, local ROIs corresponding to the stimulated left M1 and derived from >80% E-Field strength model, were used to reconstruct the signal directly evoked by TMS (3). From the preprocessed data, a minimum norm-based (MNE) reconstruction was first estimated (4). This was followed by the implementation of a novel estimator with noise and distributed source constraints to minimize crosstalk leakage from any other sources than those from our ROI (5). CTF: CrossTalk Function employed for spatial filtering ; FDI: First Dorsal Interosseous; MNE: Minimum Norm Estimator; TMS-EEG: concurrent Transcranial Magnetic Stimulation with ElectroEncephaloGraphy.

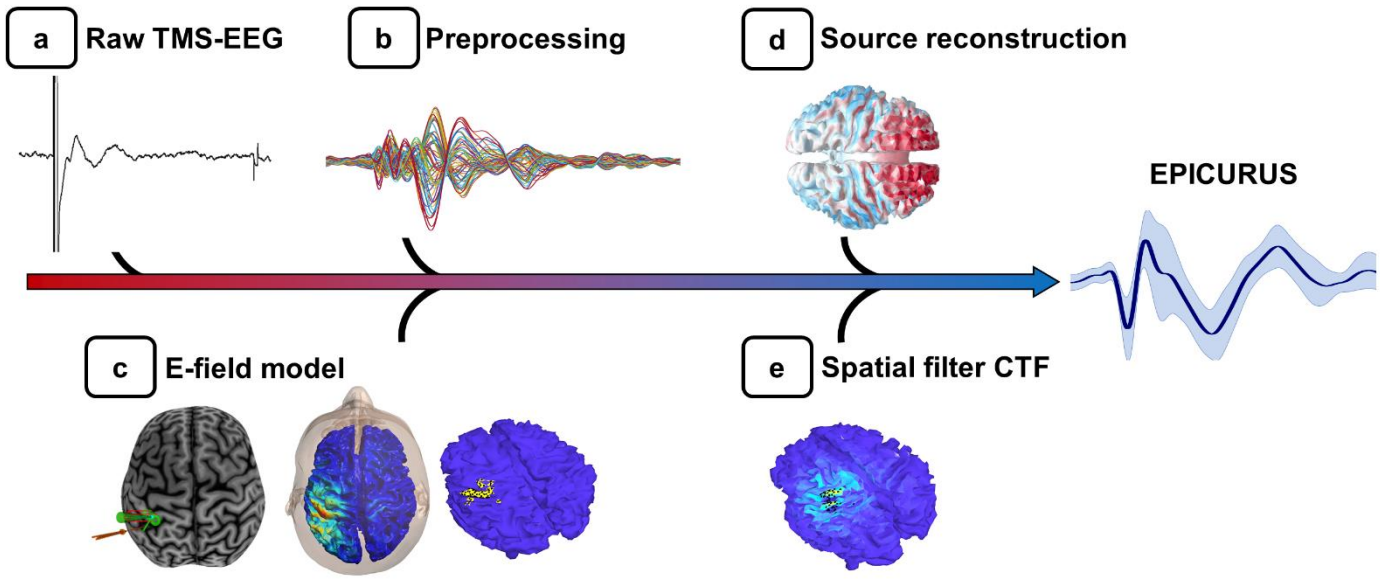
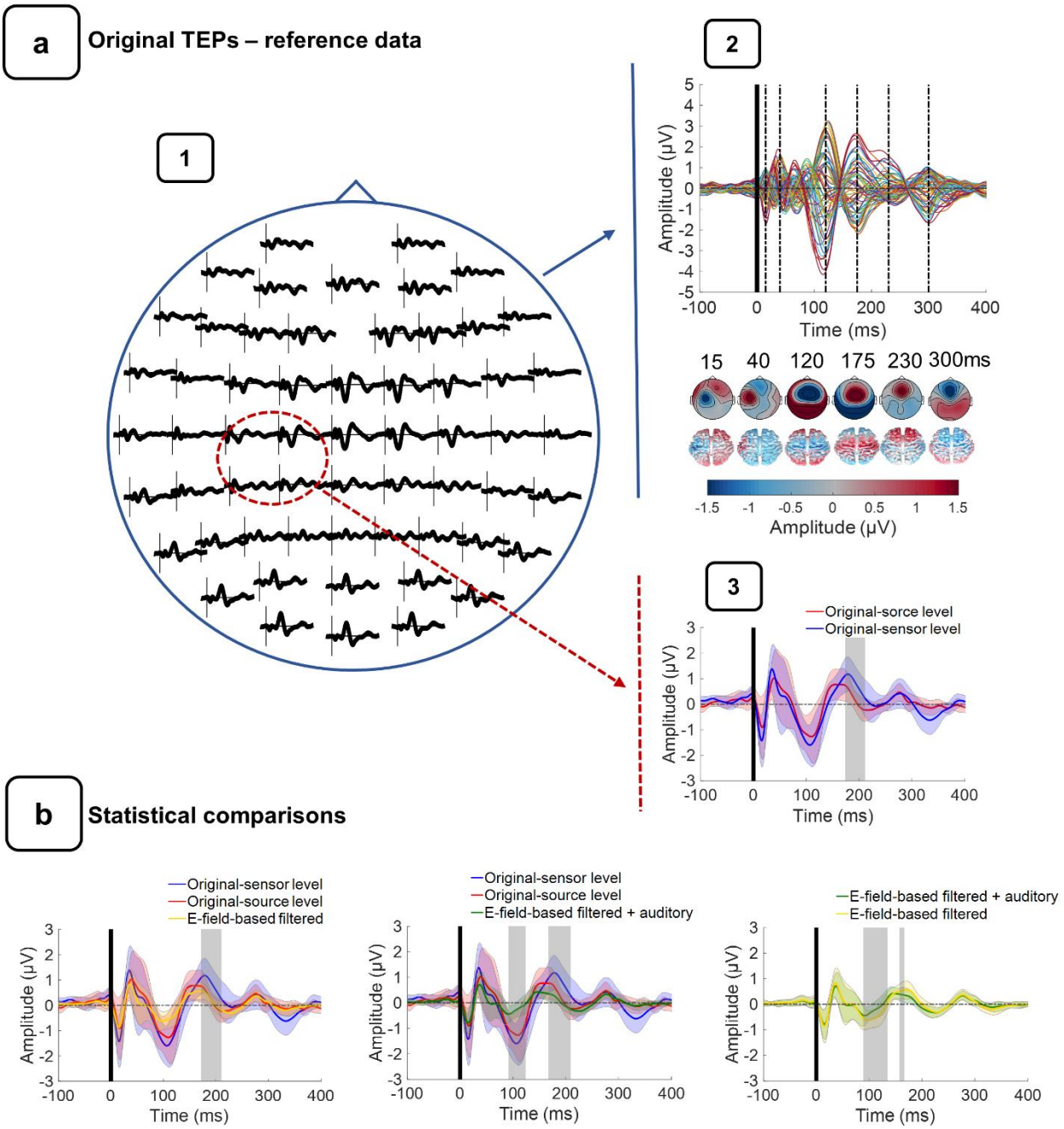


Figure 2. **Group average results**. **(A) Reference data**. **1**: Sensor-level time-course across the 64-channels corresponding to "reference data" TEPs. **2:** Sensor-level Butterfly plot (superior panel) and topographic sensor and source source-level projection (inferior panel) across the 64-channels corresponding to "reference data" TEPs. **3**: Sensor-level "reference data" TEPs from a cluster of electrodes under the stimulated left M1 (mean of C1,C3,CP1,CP3; sensor-level original; blue trace), and from the region most impacted by TMS (ROI) using a standard MNE (source-level original; red trace). **(B) Filters' validation**. **1**: Statistical comparisons of original (sensor- and source-level; blue and red traces respectively) and E-field-based filtered TEPs (yellow trace). **2**: Statistical comparisons of original (sensor- and source-level; blue and red traces respectively) and E-field-based filtered TEPs combined with auditory constraints (green trace) **3**: Statistical comparisons of filtered (with and without constraints; greed and yellow traces respectively) TEPs. Group-level source space data are visualized by projecting group-averaged signals into a standard MNI152 mesh. TEPs group mean and standard deviation across subjects are showed as a thick centered line and a background shaded line, respectively. Vertical grey bars highlight statistically significant clusters ($p<0.05$).


a
Original TEPs – reference data
1
2
3
Amplitude (μV)
Time (ms)
15 40 120 175 230 300ms
-1.5 -1 -0.5 0 0.5 1 1.5
Amplitude (μV)
Original-sorce level
Original-sensor level
b
Statistical comparisons
Original-sensor level
Original-source level
E-field-based filtered
Original-sensor level
Original-source level
E-field-based filtered + auditory
E-field-based filtered + auditory
E-field-based filtered

4.2. Experimental section 2 – Towards a better understanding of faint transcranial magnetic fields for precise neurotherapeutics

For more than three decades now, Transcranial Magnetic Stimulation (TMS) has been developed as a tool to either suppress, boost, interfere or modulate cortical brain activity. Despite being increasingly employed in experimental and clinical applications, our comprehension of the local cellular and neurochemical mechanisms of action and their relation with dose-dependent modulatory responses remain very incomplete. Along with different factors with bearing on individual dose-response effects, notably, the number and periodicity of the TMS sessions, stimulation parameters such as intensity and frequency, combined and interacting with anatomical and physiological features uniquely characterizing each individual, the intensity at which TMS is applied (a proxy of individual E-field peak and distribution), its ability to influence primary activated structures and affect the net excitatory/inhibitory output has not been necessarily well-explored. Consequently, the modulatory effects of low or weak intensity stimulation with a potential to impact human brain activity in different ways may have simply been unnoticed or disregarded.

Following the content of our first systematic review (see Manuscript 1 in Experimental Section 1), one of the key challenges to be able to extend the uses and improve the outcomes of current brain stimulation is to reach a better understanding of the neurophysiological mechanisms underlying TMS evoked activity. TMS pulses have the ability to depolarize the resting membrane potential of targeted neurons towards its firing threshold, igniting an action potential[13]. In human studies TMS intensity on a given cortical region, for example motor or visual is generally indexed to the lowest intensity able to induce an objectively or subjectively measurable physiological output of given amplitude/strength. Notably, studies have relied on the intensity required to evoke a ratio of motor-evoked potentials (MEP) or a cortical transcranial-evoked (TEP) potentials of an arbitrarily established minimal amplitude in the primary motor regions (M1) involving corticospinal responses in a hand muscle. Similarly, in retinotopically organized visual areas V1-V4, visual thresholds have been traditionally computed with a binary thresholding approach by which the minimal TMS intensity needed to elicit a given ratio (seen/unseen) of subjectively reported phosphene percepts is estimated [55].

Paradoxically, in both of such endeavors, the need of voltage current change (higher than 75-100V/m) of high intensity has been taken for granted. Yet, in contrast with the former, much weaker levels of transcranial electric stimulation (TES) with voltage density changes (± 0.5V/m) have been able show their ability to influence neocortical activity, at levels that even if considered sub-threshold for conventional TMS studies were found to provide phase processing, entrainment and a temporal bias of spikes in ongoing signals. Moreover, very weak pulsatile magnetic fields with peak magnitudes of approximately ±0.5V/m) applied to rodent models *in vivo* were able to influence online electrophysiological evoked spiking activity in directly targeted and anatomically interconnected cortical structures. A 10-minute session of 10 Hz repetitive TMS at very low intensity (10mT output) has also shown to induce long-lasting hyperpolarization of the resting membrane potential in pyramidal cortical neurons along with sustained reductions in synaptic activity and spontaneous firing. Moreover, stimulation at a slightly stronger intensity (10-15V/m) has shown signs of cortical plasticity in animal models. Similarly, weak magnetic pulses preceding a supra- threshold TMS pulse on M1 have

been consistently used in short intracortical inhibitory (SICI) protocols as pre-conditioning stimuli (CS) and delivered 1-5ms before the test stimulus (TS) onset, showed decreases in motor evoked potential (MEP) amplitude. Such an inhibitory short-lasting effect seems to reflect the combination of axonal refractoriness and 'shunting' and/or synaptic inhibition of excitatory populations, possibly even if not directly demonstrated mediated by inhibitory GABAergic interneurons with lower firing thresholds. On such basis, it has been hypothesized although not yet fully made into direct scientific evidence that weak magnetic fields could potentially tap, entrain or modulate selectively on intracortical inhibitory GABA mediated circuits and mechanisms.

To shed further light into this question, we designed and conducted three consecutive TMS-EEG experiments in the primary motor cortex (M1) of healthy human participants. We first explored (Manuscript 3) some local direct correlates of activity recorded with cortical EEG, and the dose-response relationship between TMS intensity and induced oscillatory brain activity. The former served (Manuscript 4) to compile input-output curves at increasing TMS field strength. Finally, we explored (Manuscript 5) the short-term transient modulatory impact (*offline effects or after effects*) following the delivery of weak TMS repetitive patterns.

The first experiment was carried out in a substantial cohort of n=18 healthy adult participants. Details on the theoretical and conceptual framework, the methods, analytical strategy and outcomes can be consulted in **Manuscript 3**. Briefly, concurrent TMS-EEG was used on the left (dominant) primary motor cortex (M1) to record the neural impact of single-pulse and repetitive rhythmic (20 Hz) brief bursts on oscillatory activity during stimulation (*online*) at very weak intensity (Li-TMS, 3%MSO, ~5-10V/m) compared to high intensities (Hi-TMS, 60%MSO, ~100-150V/m) conventionally employed in human TMS experiments. We specifically tested if such very weak magnetic pulses could elicit changes in cortical EEG activity, modulate or perturb ongoing oscillatory frontal M1 activity by altering local synchronization or via oscillation entrainment generated at the locally natural frequency. To this end we examined the presence and amplitude of transcranial evoked potentials (TEP), differences in the magnitude of spectral perturbations (ERSP), assessed for signs of oscillatory entrainment and changes in phase coherence (ITPC). We also assessed the pre-TMS phase dependency of such measures as a proxy of 'brain state-dependent' effects. As expected, high-intensity conventional TMS stimulation induced classical TEP responses and phase-entrainment effects on scalp EEG measures, supporting the soundness of the experimental design and technical approaches applied to reveal TMS neuromodulatory impact.

Manuscript 3: *Corominas-Teruel, X., et al., (2025). Oscillatory biomarkers of Transcranial Magnetic Stimulation impact at very weak-intensity: a TMS-EEG study.*

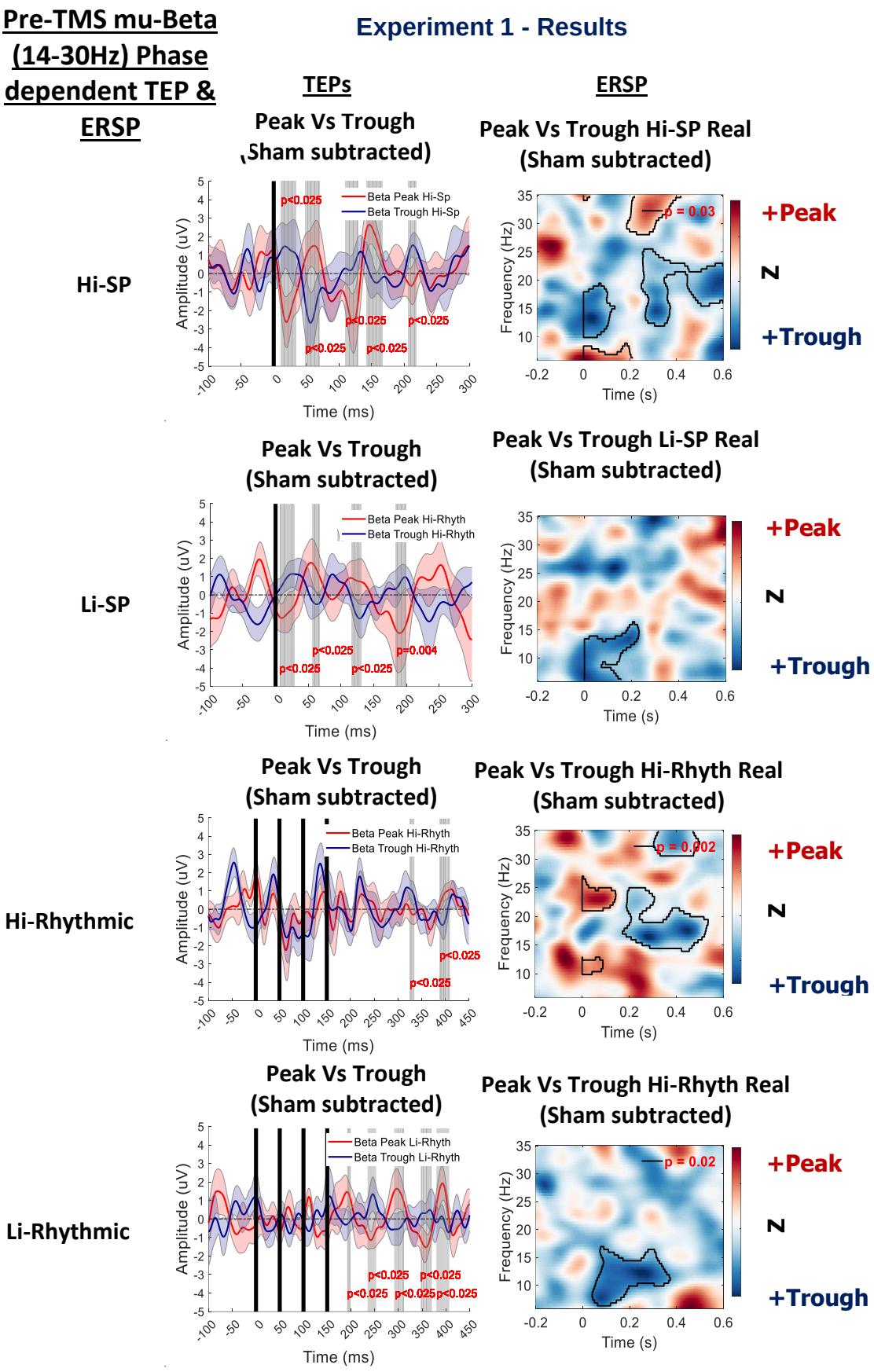


Figure 26. Summary of the scientific contributions presented in Manuscript 3. Phase-dependent TMS-EEG oscillatory signatures of high intensity (60%MSO) and low intensity (3%MSO). A detailed and complete set of results with regards to evoked activity, oscillatory perturbation and phase synchronization can be found in Manuscript 3.

In contrast, very weak intensity TMS bursts failed to reveal clear signs of TEP on cortical EEG measures neither the modulation of ERSPs or ITPCs. Yet, our analyses revealed significant increase of alpha oscillatory activity when TMS was aligned to local troughs of pre-TMS μ-beta oscillations. This last outcome suggests that neural systems could be sensitive to very weak stimulation patterns if 'brain-state' dependent measures are used to evaluate their effect. Moreover, alpha activity in the sensorimotor systems has been classically associated with inhibitory firing (see Gating by inhibition theory in 1.1.3 Communication by synchronization section of the introduction). Hence we here hypothesize, but not demonstrate, that such evoked modulatory responses could emerge from a selective recruitment of GABAergic neurons (Figure 26).

The *second experiment*, included this time a small selected cohort of n=4 healthy participants and should be considered as preliminary. Details on the theoretical and conceptual framework, the methods, analytical strategy and outcomes can be found in **Manuscript 4**. Concurrent TMS-EEG recordings were used to characterize on the left primary (dominant) motor cortex (M1) the impact of repetitive rhythmic (20 Hz) TMS short 4 pulse bursts at 20 Hz stimulation on cortical oscillatory activity at increasing levels of TMS intensities relative to each individual motor threshold (rMT) respectively at 10%, 30%, 50%, 70% and 90%rMT. We specifically assessed the ability of each TMS bursts intensities to perturb and/or to entrain oscillations on the left M1 exploring Event Related Spectral Perturbation (ERPS) and Intertrial Phase Coherence (ITPC), together with TMS evoked potentials (TEPs). Our data provide first empirical evidence that TMS at weak intensities, hence way below near motor threshold intensities regularly used in TMS research (usually at 90%rMT), can evoke and modulate local neural activity in M1. We also observed classical signatures of spectral perturbation (ERPS) and intertrial Coherence (ITPC) at intensity at 70% and 90% of the individual rMT. Surprisingly, TMS bursts delivered at 50% of the resting motor threshold, which are not commonly employed for neuromodulation purposes, demonstrated signs of frequency-specific perturbation and phase-synchronization. Furthermore, in our study, TMS bursts delivered at 30%rMT yielded to a short-lasting (200-400ms) event of local beta phase desynchronization across trials (hence beta ITPC desynchronization) following the TMS 20 Hz bursts (4-pulses).

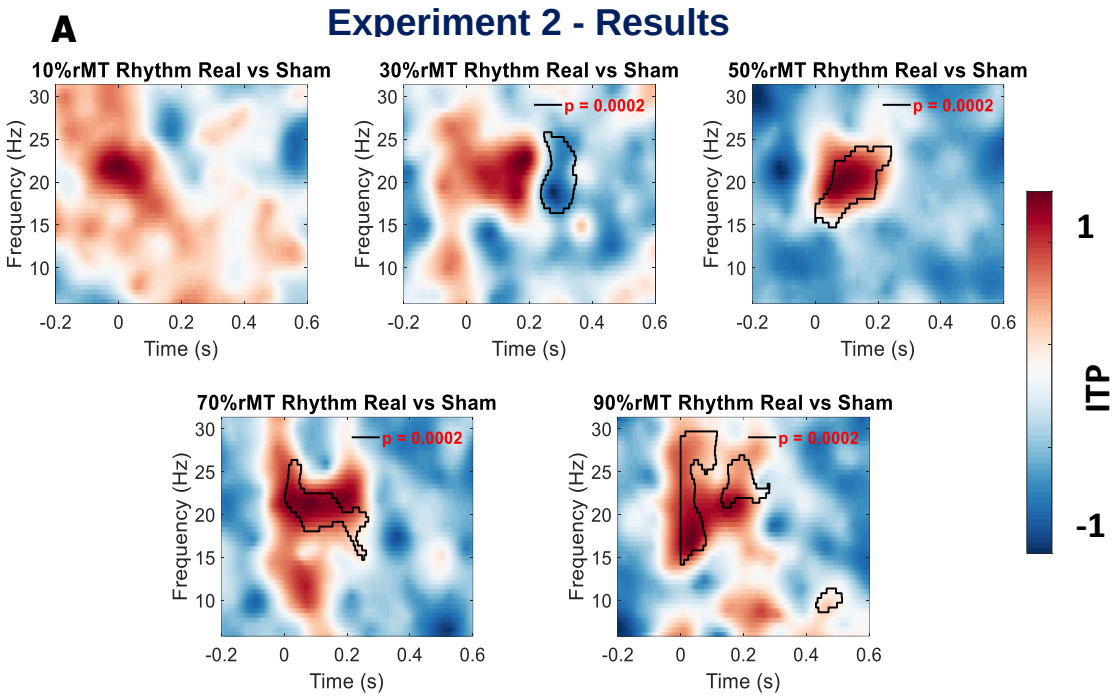
Thus, 30%rMT stimulation yielded different spectral effects respect all other conditions stimulating at superior relative intensities (50%, 70% and 90%rMT), suggesting that between 30 and 50%rMT TMS intensity, mechanisms of action under TMS may differ. Beta desynchronization after a TMS pulse has been previously reported in TMS-EEG pharmacological studies. Indeed, GABAAergic and GABABergic drugs enhanced the late beta desynchronization after single-pulse TMS, suggesting a direct link between increased local GABAergic activity and beta desynchronization. Therefore and in line with prior evidence reported in the preceding manuscript of this section, we here hypothesize that weak magnetic fields, at less than the half of the resting motor threshold (hence approximately below the half of the conventional intensity having shown effective modulation with repetitive TMS), could predominantly engage inhibitory populations, as shown by the here recorded patterns online (during TMS) alpha synchronization followed by offline (post-TMS) beta desynchronization in the localized area neighboring the primary motor cortex. Despite our results might align with those previous interpretation, our results do not directly inform about an inhibitory effect of weak magnetic fields, only suggest a modulation of EEG power desynchronizing beta frequency-band oscillators (Figure 27.A).

Manuscript 4: *Corominas-Teruel, X., et al., (2024). Entrainment with subthreshold non-invasive transcranial stimulation of the human primary motor cortex : A TMS-EEG pilot study using from very-weak to near-threshold intensity pulses.*

Finally, the third *experiment*, included again a very small and selected cohort of n=4 healthy adult participants. Extended details on the theoretical and conceptual framework, the methods, analytical strategy and outcomes can be found in **Manuscript 5**. In this preliminary work, we attempted to explore the potential of weak magnetic pulsed fields delivered as a long repetitive TMS pattern to lastingly modulate corticospinal excitability. As proof-of-concept, we implemented a study with concurrent TMS-EEG combined with EMG recording of the hand muscles, where we tested the effects of 10Hz rTMS delivered at intensity levels well below their rMT (subj1=10%, subj2=10%, subj3=10% and subj4=50% of the individual rMT) on different measures cortical and corticospinal excitability using MEPs outputs from a right hand muscle as readouts. In humans, this same 10Hz TMS frequency has demonstrated the ability to enhance cortical excitability when delivered at conventional intensities in repetitive protocols (1600 pulses, 10Hz inter-pulse interval, 40s inter-burst-interval), whereas, in rodent work by our team the delivery a very similar 10 Hz regime applied to the somatosensory cortex (barrel cortex) resulted in overt signs of suppression according to intracellular recordings, single units and local field potentials (LFP). The principal methodological difference between our rodent studies and the human rTMS studies relies on the intensity levels used. While on animal models intensity of induced electrical fields is 0.1-15V/m, in humans is approximately 70-140V/m when employed at conventional levels of intensity. To explicitly test whether the differences in cortical post-TMS facilitation or suppression could be done by the levels of intensity, participants underwent 28 minutes (1600 pulses, 40 pulses per train, 40s intertrain-interval) of 10Hz rTMS delivered to the left (dominant) primary motor cortex (Figure 27.B).

Measures of corticospinal excitability were recorded prior and immediately following (offline effects or after effect) the stimulation. Three of the participants were stimulated at 10%rMT, whereas in a fourth subject stimulated the same stimulation was delivered the 50%rMT. We assessed changes in corticospinal excitability with single pulse MEP (Sp-MEP) and shifts in intracortical modulation with paired pulse protocols such as Short Intracortical Inhibition (SICI) and short Intracortical Facilitation (ICF). Recordings from the group that received 10rMT% rTMS (n=3) explored at the group level, revealed changes in SICI and ICF amplitude compatible with a decrease of corticospinal excitability. In contrast, a control subject who received higher intensity rTMS even if still well below threshold levels (50rMT%) showed an increase of corticospinal excitability. At this stage, our small sample size limits our ability to decipher as intended weak rTMS (10% rTMS) is as we hypothesize selective for engaging net effect on inhibitory systems (either modulating GABAergic or Glutamatergic neurotransmission). Our results, highlight the potential role of intensity as a critical factor to induce plasticity changes according to their TMS levels of magnetic fields strength, while weak fields might have a predisposition to inhibit, higher intensities will facilitate corticospinal excitability increase.

Manuscript 5: *Corominas-Teruel, X., et al., (2024). May repetitive Transcranial Magnetic Stimulation at 'weak' intensity transiently modulate motor excitability? Preliminary evidence from a few cases.*



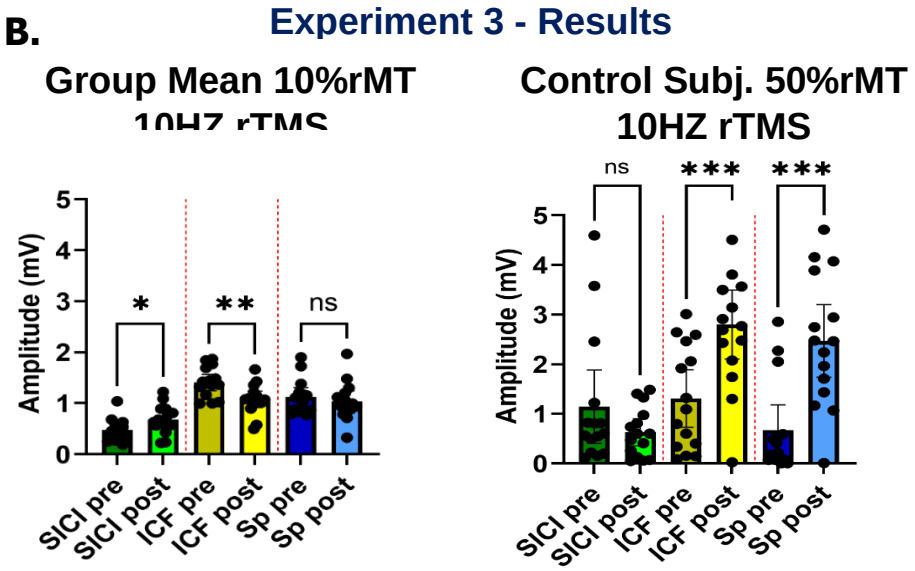


Figure 27. Summary of scientific contributions in Manuscript 4 and 5. A. Illustration of TMS-EEG oscillatory phase synchronization signatures (ITPC) at ascending levels of TMS magnetic fields strength. Extended results of evoked oscillatory activity and synchronization can be found in Manuscript 4. **B.** Results of corticospinal excitability previous and following a repetitive 10Hz stimulation protocol at different levels of TMS relative intensity.

4.2.1 Manuscript 3: Oscillatory biomarkers of Transcranial Magnetic Stimulation impact at very weak-intensity: a TMS-EEG study

Oscillatory biomarkers of Transcranial Magnetic Stimulation impact at very weak-intensity: a TMS-EEG study

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ABSTRACT

Background: Very weak transcranial magnetic stimulation (TMS, 10 mT, ~0.05-10 V/m) has been explored in animal models showcasing potential for effective neuromodulation. However, the physiological effects of these type of pulsed fields remain rather unexplored in humans. **Objective**: We here aimed to characterize the neural effects of very weak TMS pulsed fields (~6V/m, ~6% of the resting motor threshold, rMT) and explore their ability to evoke and/or modulate local oscillatory activity generated on human primary motor regions. **Methods**: Neuronavigated TMS was employed in a cohort of healthy participants (n=18) to deliver *single pulses* and *short bursts* of *rhythmic* (20 Hz) TMS at very weak intensity and at conventional levels (here referred as high intensity) usually employed in human applications (~113V/m, ~77% rMT), to the left primary motor cortex (M1). In parallel, their potential to evoke brain activity such as Transcranial Evoked Potentials (TEPs), and to entrain or modulate local ongoing oscillations was explored with scalp EEG recordings. **Results**: Both, single TMS pulses and 4-pulse rhythmic TMS beta (20 Hz) bursts delivered at conventional intensity elicited consistent Transcranial Evoked Potentials (TEPs) and beta-synchronized Event Related Spectral Perturbations (ERSP) around the left M1 area. Most interestingly, very-weak-intensity TMS modulated in this same area oscillatory activity at a mu-alpha frequency (7-13 Hz) only for rhythmic TMS bursts time-locked to -the troughs of the local ongoing beta oscillations. **Conclusions**: Our data provide first time support for the modulation of oscillatory signals with TMS delivered at an unprecedentedly weak intensity on the human primary motor cortex. This evidence enriches current knowledge on the impact of weak pulsed magnetic fields in humans, paving the way for future neurotherapeutics with a new generation of portable and autonomous low-intensity multichannel TMS devices.

Keywords: Neuromodulation, Low-intensity transcranial magnetic stimulation, TMS-EEG, Oscillatory entrainment, Brain-state dependent stimulation.

HIGHLIGHTS

1. Very weak-intensity 20HzTMS patterns evoked significant mu-alpha central activity (7-13Hz) particularly when rhythmic bursts were aligned with local troughs of central cortical beta activity.
2. Conventional high-intensity single pulse and beta 20Hz TMS patterns elicited consistent TMS evoked cortical potentials and beta-synchronized local primary activity.
3. Fulfilling established criteria to define entrainment, the effects driven by high-intensity TMS to the primary motor cortex proved to be TMS pattern-dependent and oscillation phase-dependent.
4. Differences featured by very weak vs. conventional intensity TMS on entrained oscillatory activity strongly suggest that these operate on distinct physiological mechanisms.

INTRODUCTION

Transcranial Magnetic Stimulation (TMS) is a non-invasive brain stimulation (NIBS) technique widely employed in experimental neurosciences and neuropsychiatric applications. This stimulation modality is predominantly delivered at intensities (~100-150V/m), individually adapted to each participants' susceptibility to generate a measurable physiological output[1]. In the primary motor cortex, TMS intensity is tailored based on the resting Motor Threshold (rMT), defined as the strength of single TMS pulses required to elicit with 50% probability, motor evoked responses (MEP) with a minimal established amplitude (usually 50μV peak-to-peak) on a hand muscle[2]. Likewise, the Phosphene Threshold (PT) defines the lowest TMS intensity to be delivered occipitally to retinotopically organized primary visual area V1 necessary to elicit, according to subjective reports, a detectable percept at least in half of the attempts [3–5].

Traditionally, stimulation patterns at intensities well-below such cut-off threshold levels, hence unable to evoke TMS evoked brain potentials (TEPs) on electroencephalography (EEG) recordings [6] have been assumed to be unable to evoke significant online changes of neural activity in the human brain. However, evidence in the field of neuromodulation provides support for the possibility that much weaker intensities, likely operating on different mechanisms than those classically attributed to TMS at conventional strength, could affect brain activity. For example, neuromodulation with transcranial Electrical Stimulation (tES), another family of NIBS technologies employed at intensity levels of ~0.4 to 0.6V/m is unable to directly evoke *per se* measurable physiological effects such as peripheral motor evoked potentials or cortically generated phosphene percepts; Yet, it can influence brain excitability and induce phase-entrainment effects in thalamocortical networks mediated by ongoing oscillatory brain intrinsic activity [7–11] and ephaptic coupling[12–14]. Likewise, *very low* pulsatile magnetic fields applied to rodent models *in vivo* at peak magnitudes of ~0.5V/m have recently shown to influence *online* evoked spiking in neurons from locally targeted and interconnected structures[15,16]. Similarly, evidence generated in rats by our own team has recently shown that a 10-minute long 10 Hz repetitive TMS session at very low intensity (10mT output) induces a long-lasting

hyperpolarization of the resting membrane potential in S1 pyramidal neurons (barrel cortex), along with sustained reductions in synaptic activity and spontaneous cell firing[17]. Moreover, still in rodents, low intensity TMS stimulation (0.01-0.07T, ~0.05-15V/m), well below the intensity levels at which TMS is conventionally used in humans, has shown to induce cortical plasticity[18–21].

In spite of evidence from animal models, surprisingly, to date only two studies have systematically addressed in humans the effects of repetitive TMS at significantly lower intensity levels than those conventionally employed in human noninvasive stimulation. These reported that weak intensity rhythmic TMS patterns (35 or 50V/m) at 10 Hz delivered to the primary visual cortex elicited *online* (i.e., during TMS) signatures of parieto-occipital alpha synchronization[22], whereas repetitive rhythmic alpha stimulation (20-pulse bursts fora total of 25 bursts) to the primary motor cortex reduced *offline* (i.e., post rTMS) corticospinal MEP excitability[23]. On such a basis, it has been hypothesized -although not yet fully demonstrated- that low-intensity magnetic pulsed fields hold a potential to selectively engage intracortical inhibitory mechanisms in the human motor cortex. Moreover, since human brain oscillatory states -measured with EEG- reflect transitory changes in intracortical excitatory/inhibitory balance, the combination of TMS- and EEG seems uniquely suited to probe at even lower intensities the suspected local neuromodulatory effects of weak intensity stimulation reported above[24]. In such a context, and aiming to shed further light into the potential effects of low intensity TMS and extend current evidence of its neuromodulation effects to healthy humans and lower strengths as comparable as possible to those verified in rodents, here we experimentally searched and characterized *online* EEG signatures of very weak magnetic pulsed fields (estimated cortical current density ~6V/m, 3% MSO, maximum stimulator output, ~6%rMT). To this end, MRI-neuronavigated TMS-EEG recordings were used to assess the effects of active vs. sham single pulse TMS and rhythmic (20Hz) TMS bursts, delivered to the left primary motor cortex (M1). Importantly, we compared the effects of weak-intensity magnetic stimulation with those achieved with conventional TMS intensity (referred to in this manuscript as 'high-intensity') (~113V/m, 60%MSO, ~77%rMT),

characterizing their local impact on evoked activity and oscillatory entrainment[5,25–32]. Based on evidence reported in rodent studies, we hypothesized that very weak magnetic fields, even if unlikely to produce consistently TEPs, may impact/modulate oscillatory activity by transiently acting on rhythmic processes. The topic is relevant and timely, since solid evidence on effective neuromodulation with weak-intensity magnetic pulses could pave the way to therapeutic applications with energy-autonomous multichannel TMS stimulation devices able to operate on extended cortical networks and wide-spread oscillatory signatures [33].

MATERIAL AND METHODS

Participants

Eighteen healthy volunteers (n=18, mean age ±SD = 26.1 ± 5.8 years; 10 females / 8 males), all right-handed according to a standard handedness inventory test[34], free of neurological, or psychiatric symptoms and with normal or corrected-to-normal vision were recruited for this study. All participants satisfied international TMS and magnetic resonance imaging (MRI) safety criteria[35,36] and provided informed consent prior to the study. The research protocol respected the Declaration of Helsinki, French legislation regulating good clinical practices (ICH-E6, R2) and received the approval of a local ethical committee (*Comité de Protection des Personnes, Ile de France I*). No participant reported serious discomfort or adverse events to TMS, which were in any case, systematically evaluated using a standardized international questionnaire suited for their identification[37].

Experimental design

Participants took part in two different sessions. In the first one, a 3D-T1-weighted MRI scan (3T Siemens MP2RAGE, Flip angle=4, TR=5000ms, TE=3.24ms, slice thickness=1mm, isovoxel) was recorded for subsequent TMS neuronavigation and computational modelling of E-

field distribution. In a second session, TMS was applied to the participant's left M1, while 64 electrode scalp EEG was concurrently recorded. Participants were exposed to a total of 4 stimulation conditions: *Single-pulse* TMS, and short *rhythmic* 20Hz TMS, each delivered at very weak intensity (*Li-TMS*) and at conventional High-Intensity (*Hi-TMS*) (see below for further details). Since the effects of each TMS condition were evaluated in its active and sham modalities, each participant was assessed on a total of 4 active TMS conditions (and their 4 associated sham scenarios). Importantly the sequence of the TMS blocks was randomly counterbalanced across participants to avoid order bias effects.

Transcranial Magnetic Stimulation (TMS)

Stimulation was applied over the left M1 with a 70 mm figure-of-eight coil connected to a biphasic rTMS stimulator (Magstim Rapid2). The left M1 was identified as the optimal cortical hotspot on which TMS pulses elicited Motor-Evoked Potentials (MEPs) over the first dorsal interosseous (FDI) muscle of the right hand index finger. During the whole session, TMS coil position was tracked using an MRI-based frameless stereotactic neuronavigation system (Brainsight, Rogue Research) kept within a 2mm radius from the center of the established targeted M1 hotspot site. Prior to the start of the session, we measured and documented for each participant their resting Motor Threshold (rMT). To this end, TMS single pulses were delivered over the M1 hotspot corresponding to the right FDI hand muscle while we recorded the amplitude of the evoked-MEPs (Signal 5.0, Cambridge Electronic Design Limited, Cambridge, UK). Starting at 65% of maximal stimulator's output (from now on MSO), TMS was delivered at varying intervals between ~4-8 secs, with intensity increases/decreases by steps of 1-5% of the MSO, depending on the consistency and amplitude of evoked MEPs responses. The individual rMT was defined as the lowest TMS intensity able to induce at least 5 out of 10 MEP (50% responses) with a minimal peak-to-peak amplitude of 50μV.

For high-intensity blocks, TMS intensity was set at a fixed level of 60% of the MSO (68.8 dI/dtmax [A/µs])[38] which corresponded to an average of ~6% rMT. For very weak-intensity TMS blocks, we established an intensity of 3% of the MSO (3.44 dI/dtmax [A/µs]), which was the lowest level eliciting consistent pulse output with our equipment and corresponded to ~77% rMT for the tested cohort (see Supplementary Materials 1). For each experimental condition (Hi-Sp TMS, Li-Sp TMS, Hi-Rhythmic TMS, Li-Rhythmic TMS), two rounds (blocks) of 60 trials involving either single TMS pulses (Sp- TMS) or TMS rhythmic bursts (Rhythmic TMS) were administered. The latter consisted in 4 pulses of rhythmic TMS delivered at 20 Hz (inter-stimulus interval (ISI) of 50ms with a total burst duration of 150ms) intended to synchronize (or 'entrain') cortical oscillators on the targeted on M1 at this same frequency. Each block included 40 active pulses and bursts and 20 randomly-embedded sham pulses or bursts resulting in a total of 120 trials per participant and condition, with an inter-trial interval (ITI) of 6 secs (hence 80 active and 40 sham pulses/bursts per participant) for each of the conditions (see Supplementary Materials Figure 1 for a schematic illustration of the procedure). Sham pulses/bursts were delivered with a second rTMS machine (Magstim Super Rapid^2) using an identical figure-of-eight TMS coil (70 mm double coil) oriented perpendicularly and placed right above the active coil, so that an ineffective magnetic field was projected away from the scalp.

TMS-EEG data acquisition

During each session, EEG was continuously recorded with a set of two TMS-compatible EEG pre-amplifiers (actiChamp, Brain Products) attached to 64 active electrode grid (ActiCap snap), mounted on an elastic cap following International 10–10 EEG system positions. FPz and Fz served as *ground* and *reference*, respectively. Impedances were periodically monitored and kept during the whole session below 5kΩ and signals were sampled at 25kHz to better pinpoint the onset and offset of TMS pulse artifacts. To limit TMS-related auditory evoked EEG components participants wore foam earplugs through which white noise was continuously played

(ER-3C insert earphones, ETYMOTIC Research) at a volume tailored individually to ensure participants were unable to notice the TMS clicking sound, particularly when pulses were delivered at high intensity (60% MSO). To minimize muscle and eye movement artifacts, participants were seated in a comfortable armchair with their heads on a chinrest, ensuring stable position and asked to remain relaxed and with their gaze fixated around a white cross (2x2º degrees of angle) displayed at the center of a computer screen, placed at 57 cm from the participant eye´s canthi.

Figure 1. Schematic illustration of TMS-EEG data management procedures

TMS-EEG pre-processing

Signal preprocessing was performed with MATLAB (The MathWorks Inc., Natick, MA, USA)using EEGLAB software (UC San Diego, La Jolla California, USA;[39]) and its TMS–EEG signal analyzer plugin (TESA;[40]). Every dataset was preprocessed individually and visually inspected after every step (see Figure 1 for a general overview of methods, and Supplementary Materials Figures 2.1 and 2.2 for a schematic illustration of the EEG pre-processing pipeline). EEG data were epoched around the TMS pulse (or the 1st pulse of each burst, [-1.200ms,1.200ms]). TMS-pulse electromagnetic artifacts were removed and zero-padded ([-2ms,10ms]) and the recharging artifact removed and interpolated using a spline cubic function[41]. Following, datasets were downsampled to 5KHz and decomposed with Independent Component Analysis (ICA, using EEGLAB ‘runica’ algorithm, 60 maximum components). 'Bad' channels were identified and labeled and excluded from the ICA. Ocular motion components were also singled out as artifacts based on well-established guidelines[42]. On average 9±4 (mean±SD) components were rejected. Next, data was high-pass filtered with a fourth-order backwards Butterworth filter (2Hz). The SOUND algorithm (employing the simplified lead field based on a spherical-head-3-layer model) was used to suppress recording noise whereas the SSP-SIR spatial filter cancelled remaining TMS-evoked muscle artifacts[43,44]. Missing (i.e., zero-padded)

signals were cubically interpolated, and a second ICA was computed to remove remaining residual muscle-artifacts. Datasets were bandpass filtered with a fourth-order Butterworth filter ([2-85Hz], downsampled to 1Kz, baseline corrected and re-referenced to an average of all channels.

E-field informed TMS-EEG spatial filtering

To improve the signal-to-noise ratio of our dataset, we implemented a spatial filter that isolated activity emerging from the primary targeted region (the left M1 hotspot for the FDI muscle) and minimized the contribution of multisensory responses (particularly likely evoked during the high-intensity TMS condition at 60%MSO). Since the TMS coil position on the precentral gyrus M1 region differed slightly across participants, this procedure allowed for individualized EEG signal reconstruction. To this end, we first modeled the electrical field (E-field) induced by TMS on each participant's head and brain volumes. Tetrahedral Finite Element head Models (FEM) were generated from each individual structural T1-weighted MRI scans using the '*charm'* routine of SimNIBS4.0[45,46]. Electrical fields were then estimated assuming a quasi-static regime[47–49]. TMS coil spatial coordinates [X, Y, Z] for coil simulation were individually extracted from real-world coil position documented during the experimental session with MRI-based neuronavigation software. Then E-field distribution models were computed with a Magstim figure-of-eight 70 mm coil (D70) powered by a MagstimRapid^2 (114.7 dI/dtmax [A/μs] = 100% MSO)[38]. This included models for each participant for the 3%MSO and 60%MSO TMS intensity conditions employed during our experiments (see Supplementary Materials 2 for details).

Based on the modeled current field distribution, we built an individual spatial filter with special weighting constraints fulfilled by E-field outcomes. The procedure was based on a previously developed framework for the design of crosstalk functions for spatial filtering applied to EEG/MEG data (DeFleCT)[50]. The implemented procedure employed the tools SimNIBS

4.0[45], ISO2MESH[51], the Helsinki BEM framework[52], EEGLAB[39], TESA toolbox[40], DeFleCT framework[50] and operated as follows: From the E-field simulations we first outlined the primary impacted cortical structure (>80% of the maximal TMS-induced E-field strength). In parallel, we computed the leadfield matrix from the derived forward models[52]. Finally, the cross-talk leakage of our sources of interest (sources within the magnetic field boundaries (>80%)) was estimated and used as a source-based spatial filter (aka cross-talk function) employing a minimum norm like estimator for every participant and applied to the EEG data delimiting pass-band sources and minimizing cross-talk leakage from other non-highlighted sources anywhere in the brain. The procedure source-informed, reconstructed and separated our EEG signals of interest (i.e., from the primarily targeted cortical source) while minimizing crosstalk from other locations anywhere else in the brain. Details of E-field simulations, ROIs and crosstalk leakage of the employed spatial filters can be found in Supplementary Materials Figure 3.

TMS-EEG data analysis and statistics.

All analyses were implemented in MATLAB, employed the Fieldtrip[53] toolbox and involved as already indicated, E-field-informed source reconstructed EEG signals. To understand if very weak-intensity stimulation was able to (1) evoke neural activity, (2) perturb brain oscillations, (3) induce local synchronization or entrainment, and (4) understand the role of different intrinsic synaptic input levels to the elicited oscillations (i.e., brain state dependent effects), we analyzed the following metrics, respectively: (1) Changes in the amplitude of TMS Trascranially Evoked Potentials (TEP); (2) Modulation of Event-Related Spectral Perturbation (ERSP), also referred to as total evoked oscillations and/or elicited oscillations); (3) Modulation of Inter-Trial Phase Coherence (ITPC); and (4) Pre-TMS phase-dependent effects of elicited oscillations (aka. phase-dependent ERSP).

For evoked activity analyses (TEP), EEG epochs representing all the trials of a given condition were first bandpass filtered ([2-45Hz]). Then, TEPs were calculated by averaging the

EEG epoched signal ([from -0.5s to 0.6s with respect to the TMS pulse onset]), separately for each experimental condition. Then, signals were baseline corrected ([-0.5s, -0.2s]). For spectral perturbation analyses (ERSP), signals were time-frequency transformed for the ([8-35Hz]) frequency range (i.e., surrounding stimulated frequency 20Hz) in steps of 0.01Hz using complex Morlet wavelets[54,55]. For local entrainment analyses (i.e., ITPC), signals were time-frequency transformed for the ([8-35Hz]) band using complex Morlet wavelets, whereas phase synchronization across trials was estimated immediately thereafter. To better understand the nature of the TMS frequency-dependent phase synchronization and discern TMS-evoked oscillatory genuine episodes from repetitions of evoked potentials, we employed the *fBOSC* algorithm to parametrize the aperiodic component 1/f ([8-35Hz]) and detect putative oscillatory episodes surviving at least 3 oscillatory cycles (95^{th} percentile threshold)[56,57]. Finally, we specifically evaluated phase-dependent ERSPs, and filtered trials according to their pre-TMS mu-alpha phase (8-13Hz) and pre-TMS mu-beta phase (14-30Hz) data. To this end, a first window of interest preceding the TMS pulse (1st TMS pulse for rhythmic burst conditions) was isolated ([-5ms, -2ms]) and Hilbert transformed to obtain the instantaneous pre-TMS phase of each epoch. In parallel, a second broader window of interest ([-400ms, -3ms]) was identified to calculate the power spectra with complex Morlet wavelets. Only individual trials exceeding 50th percentile (>50%max) of power were identified and considered for further analysis as proxies of pre-TMS oscillatory activity. We then classified trials according to their phase to retain those at the *peak* (330º-30º) and at the *trough* (150º-210º) of the ongoing oscillation. Their associated ERSPs were computed for every single condition and statistically compared. All ERSP and ITPC analyses preceded a signal Z-transformation concerning the mean and standard deviation of the entire epoch length ([-0.5s to 0.6s]) and baseline correction ([-0.5s, -0.2s]). We statistically compared filtered trials corresponding to pre-TMS mu-alpha phase and pre-TMS mu-beta phase for each active TMS condition to its associated sham (Active Hi-TMS *vs.* Sham Hi-TMS and Active Li-TMS *vs.* Sham Li-TMS). TEP analyses involved two-tailed t-tests (alpha threshold: $p<0.05$) with Montecarlo permutations (50000 iterations) to correct for multiple comparisons. The analyses of ERSP and ITPC involved nonparametric cluster-based permutation statistics (5000 iterations) to

control for false discovery rate[58]. Monte Carlo p-values were calculated via a two-tailed test (alpha cluster: $p<0.05$, alpha threshold: $p<0.05$). The time domain was set to permute from the TMS onset until the epoch end ([0ms, 600ms]). Finally, the same statistical approach was employed for phase-dependent analyses; trials were first sorted according to pre TMS alpha and beta phase, and TEP amplitudes or ERSP magnitudes evoked by a TMS pulse aligned with the *trough* vs. the *peak* of the ongoing oscillation were directly compared.

RESULTS

Analysis of evoked cortical activity: Transcranial Evoked potentials (TEP)

To test whether single pulse TMS at high and very weak intensities was able to impact cortical function, we compared the time-courses of transcranially evoked local M1 potentials (TEPs) with a focus on amplitude differences. As expected, t-test analyses performed on TEPs elicited by ACTIVE vs. SHAM single-pulse TMS at high-intensity (Hi-TMS, 60%MSO) yielded several statistically significant clusters in the time domain showing significant amplitude differences (~25ms p=0.02, ~45ms p=0.01, ~100ms p=0.009, ~160ms p=0.006, ~230ms p=0.007, ~275ms p=0.006, ~340ms p=0.02, two-tails, paired t-test) and showcasing well-established early evoked EEG signatures of TMS pulses in the vicinity of the left M1 region and the impact of late re-afferent entries. In contrast, no significant activity was evoked by single TMS pulses at very weak-intensity (Li-TMS, 3%MSO) (see Figure 2). The time-courses of TMS evoked potentials for all conditions (Hi-TMS and Li-TMS Single pulse and Rhythmic patterns) are displayed in Supplementary Materials Figure 4.

Figure 2. Single Pulse TMS evoked potentials (TEP).

Analysis of Event-related perturbations (ERSP)

A cluster analysis in the time and the frequency domains comparing the time-frequency power spectra of local M1 cortical activity tested whether TMS at high and very weak intensities was able to perturb ongoing brain oscillations. ERSP cluster-based statistics performed on ACTIVE vs. SHAM single-pulse TMS at conventional high-intensity (Hi-TMS, 60%MSO) yielded a cluster of early (~0-100ms, $p<0.0001$) elicited spectral power increase around the beta band (10-35Hz) with a longer lasting epoch (~100ms-300ms) of beta oscillatory (~20-25Hz) activity. A contrast between ACTIVE vs. SHAM High-intensity rhythmic TMS bursts revealed a power increase centered around the stimulated frequency (20 Hz) for a significantly longer period (from 0 to 300ms, $p=0.004$) than the TMS burst itself (from 0 to 150ms). On the other hand, neither ACTIVE vs. SHAM single pulse TMS nor rhythmic TMS delivered this time at very weak-intensity (Li-TMS, 3%MSO) elicited statistically significant power increases for any of the bands of the analyzed time-frequency spectrum (see Figure 3).

To further understand the nature of such impact, we dissociated between *evoked* (time-locked to TMS stimuli) and *induced* (non-time-locked to TMS stimuli) oscillations[59,60]. Interestingly, single pulse TMS at High-intensity *evoked* direct activity at ~30Hz (0-100ms, $p<0.0001$) and *induced* longer-lasting oscillations at ~23Hz (0-250ms, $p<0.0001$). Finally, none of the very weak-intensity TMS conditions tested in parallel (Li-TMS, 3%MSO) showed specific trends or significant patterns for *evoked* or *induced* oscillations (see Supplementary Materials Figure 5.1).

Figure 3. Event-related spectral perturbation (ERSP).

Analysis of inter-trial phase coherence (ITPC) and frequency-specific entrainment

By means of a cluster analysis in the time and phase domains comparing inter-trial phase coherence (ITPC) measures present in local cortical activity, we then assessed , whether TMS at high and at very weak intensities were able to synchronize the underlying cortical generators in the targeted M1 hotspot and entrain oscillations. ITPC cluster-based statistics performed on

ACTIVE vs. SHAM single-pulse TMS at high-intensity (Hi-TMS, 60%MSO) revealed clusters in the temporal and phase domains with early (~0-300ms, p<0.0001) alpha to high beta (8-35Hz) phase-synchronization, particularly with a noteworthy increase of power levels at ~30Hz. A contrast between ACTIVE vs. SHAM high-intensity rhythmic TMS bursts yielded clusters with inter-trial alpha and high-beta (8-35Hz) phase-synchronization in the vicinity of the stimulated frequency (20Hz) lasting for longer (from 0 to 300ms, p=0.004) than the TMS burst itself (0-150ms). In contrast, no phase alignment effects were found for any of the very weak TMS intensity (Li-TMS, 3%MSO) conditions (ACTIVE vs. SHAM Li-TMS single pulse, ACTIVE vs SHAM Li-TMS rhythmic bursts) (see Figure 4.A). To better disentangle between entrained oscillatory episodes and elicited repetitions of transcranial potentials (generating slow amplitude oscillations), we conducted analyses with the so-called fBOSC method in the vicinity of the TMS delivered frequency (8-35Hz). Two-tailed t-tests served to compare the number of detected oscillatory episodes for all TMS conditions vs. their respective Sham conditions in equalized samples (612 random trials per condition surrounding TMS events, (-100ms 400ms). A statistically higher number of oscillatory episodes was found for rhythmic TMS patterns at high intensity compared to Sham (t=5.30, p<0.0001) (see Figure 4.B). Further comparisons across all active TMS conditions confirmed a superior number of episodes for the high intensity rhythmic TMS condition compared to all the remaining active patterns (Hi-TMS Single pulse (t=4.04 p=0.003), Li-TMS -Single pulse (t=6.04 p<0.001) and Li-TMS -Rhythmic bursts (t=5.36 p<0.0001).

Figure 4. Intertrial Phase coherence (ITPC) and oscillatory episodes .

Mu-alpha pre-TMS phase-dependent perturbations.

To test whether TMS evoked activity and spectral perturbations were brain-state dependent according to defined pre-TMS mu-alpha oscillatory phases (here considered as a proxy of intrinsic fluctuations of M1 excitability), we performed statistical comparisons on TEP

amplitude and ERSP power for mu-alpha phase-sorted trials, contrasting values at *peak* vs. the *trough* of the ongoing oscillation for ACTIVE TMS conditions (after subtracting their associated SHAM patterns).

Statistical comparisons of TEPs amplitudes elicited by single TMS pulses at high-intensity (Hi-TMS, 60%MSO) delivered at the *peak* vs. the *trough* of the oscillation revealed statistically significant clusters in the time domain at ~90ms, ~200ms and ~235ms ($p<0.025$). Similarly, single TMS pulses delivered at low-intensity (Li-TMS, 3%MSO) also yielded statistically significant clusters at ~45ms, ~175ms, suggesting the presence of an early potential (P45) with a higher amplitude for TMS pulses aligned with the trough, compared to the peak of the ongoing alpha oscillation. However, no differences were found between this same condition and contrast for ERSPs spectral power.

The analysis of TEPs amplitude evoked by high intensity-TMS rhythmic bursts (Hi-TMS) did not reveal statistically significant clusters between phase (*peak* vs *through*) epochs, but evoked an early (~15Hz, 100ms-350ms, $p<0.03$) and a late (25Hz, 200ms-400ms, $p<0.03$) cluster in the time and frequency domain with increased spectral power, suggesting higher neuronal recruitment levels when the first pulse of the TMS burst was aligned with the *peak* of the ongoing mu-alpha oscillations. Finally, TEPs evoked by low intensity TMS rhythmic bursts (Li-TMS) elicited several statistically significant clusters in the time domain at ~175ms (15ms after the last pulse), ~250ms (100ms after the last pulse) and ~310ms (160ms after the last pulse) ($p<0.025$), with higher amplitude TEPs for TMS pulses aligned to the *peak* of the alpha oscillations. Additionally, a cluster on ERSPs analyses showing early spectral power increases at a beta band ~20-25Hz (0ms-100ms, hence during the TMS burst, $p<0.04$) indicates higher beta oscillatory power when TMS bursts were aligned with the trough of mu-alpha oscillations (see Figure 5).

Figure 5. Pre-TMS mu-alpha phase-dependent TEP & ERSP.

Mu-beta pre-TMS phase-dependent perturbations.

To further assess the state-dependency of TMS evoked activity and spectral perturbations according this time to pre-TMS mu-beta oscillatory phase, we computed statistical comparisons for TEP amplitude and ERSP spectral power on the basis of mu-beta phase-sorted trials (*'peak* vs. the *trough*) for ACTIVE TMS conditions after subtraction of their respective SHAM patterns. A comparison of TEP amplitudes for *peak* vs. *trough* sorted trials according to pre-TMS ongoing mu-beta oscillations following Single Pulse TMS at high-intensity (single pulse Hi-TMS, 60%MSO) revealed statistically significant temporal clusters at ~20ms, ~60ms, ~120ms, ~150ms and ~210ms ($p<0.025$). Comparisons involving ERSPs spectral power yielded in the time and frequency domains a ~15Hz early cluster (0ms-100ms, hence during the TMS burst, $p<0.03$) and the elicitation of a late power increase (300ms-600ms, $p<0.03$) on the ~15-25Hz band, suggestive of higher beta oscillatory power when TMS bursts were aligned with the *trough* of the ongoing mu-beta oscillations.

The analysis of TEPs amplitude elicited by Single Pulse TMS at low-intensity (Single Pulse Li-TMS, 3%MSO) resulted in statistically significant clusters in the time domain ~15ms, ~60ms, ~125ms and ~190ms. In contrast, ERSP analyses yielded a single early cluster in the temporal and frequency domains (0ms-200ms, hence during the TMS burst, $p<0.03$) showing a spectral power increase (~6-15Hz), hence supporting higher alpha oscillatory power when TMS bursts were aligned with the *trough* of mu-beta oscillations.

High intensity rhythmic TMS bursts (Rhythmic Hi-TMS, 60% MSO) elicited very modest late clusters (~330ms; 175ms after the last pulse, and ~400ms; 250ms after the last pulse) of higher TEP amplitude potentials for pulses aligned with the trough of the beta oscillation. The analysis of ERSP modulated by Hi-intensity rhythmic TMS bursts elicited early clusters in the time and frequency domains (0ms-150ms, $p<0.002$) of power increase (~12Hz and ~24Hz) for pulses delivered as aligned with the peak of ongoing beta oscillation (hence during the TMS burst), and most importantly, a later cluster (200ms-500ms, $p<0.002$) with spectral power

increases at ~20Hz when the first TMS pulse of the burst was aligned to the trough of the ongoing mu-beta oscillation.

Finally, the analysis of low intensity rhythmic TMS bursts (Rhythmic Li-TMS, 3% MSO) effects on TEP yielded several late clusters at ~190ms (40ms after the last pulse), ~250ms (100ms after the last pulse), ~300ms (100ms after the last pulse), ~360ms (210ms after the last pulse) and ~390ms (240ms after the last pulse) ($p<0.025$), with higher amplitude TEPs for potentials generated by pulses aligned to the peak of the beta oscillation. Comparisons of ERSP, also revealed a cluster showing an early (100ms-400ms, $p<0.02$) increase of spectral power for high alpha oscillatory activity, ~6-15Hz, occurring when TMS bursts were aligned with the *trough* of the ongoing mu-beta oscillations (see Figure 6).

Figure 6. Pre-TMS mu-beta phase-dependent TEP & ERSP.

DISCUSSION

The electrophysiological signatures of weak magnetic stimulation have failed to gather significant interest in the human TMS literature and evidence remains very scarce. Nonetheless, according to available evidence, there was no reason to believe that weak magnetic pulsed fields generating induced electrical cortical currents between 1-10V/m could not theoretically induce ephaptic coupling, entrain ongoing oscillatory phenomena intrinsic to neuronal systems, or perturb brain oscillations in a manner similar to transcranial electrical stimulation [7,12]. In the present study, we employed concurrent TMS-EEG to assess the potential impact of very weak pulsatile TMS fields (Li-TMS, 3%MSO, ~6V/m, ~6%rMT) on the human brain as compared to equivalent TMS patterns delivered at intensities conventionally used in human neurostimulation studies, also referred to as 'high intensity´ TMS (Hi-TMS, ~60%MSO, ~70-140V/m, ~77%rMT). To this end, we investigated the neural signatures of single pulse and short rhythmic TMS bursts generated *online* -hence during or immediately following TMS patterns- via different correlates of EEG activity recorded locally around the targeted left primary motor cortex (M1).

As expected, high intensity TMS yielded transcranial evoked potentials (TEPs) in response to TMS-single pulses and elicited time and frequency-dependent spectral perturbation and entrainment phenomena (i.e., changes in ERSP and ITPC) during 20Hz rhythmic TMS bursts. Our data support the reproducibility of frequency-dependent TMS evoked effects[26,27,29,32,61] in motor cortical systems when stimulation is applied at an intensity range used conventionally for neuromodulation in human studies and defined in this publication as ‘high-intensity TMS’ (Hi-TMS). Most importantly, very weak-intensity stimulation used in this study did not directly trigger changes of cortical activity according to classical measures of evoked activity and local synchrony (changes in TEPs amplitude, ERSP spectral power or ITPC magnitude).

Prior research has shown that spontaneous fluctuations of neocortical rhythms orchestrate the timing of information flow in an oscillation phase-dependent manner[62–64]. Accordingly, single pulse TMS at supra-threshold intensity (relative to resting M1 corticospinal thresholds) delivered at oscillation cycle phases suggestive of highly disinhibited cortical states (i.e., the trough of the mu-alpha oscillation within sensorimotor areas) facilitates neuronal input-output gain recruitment[65,66]. In our study, rhythmic 20Hz TMS delivered at ‘conventional intensity’ (referred in our manuscript as 'high intensity’ Hi-TMS) but still below resting motor threshold criteria (~77% of rMT), resulted in stronger oscillations when the onset of rhythmic TMS bursts aligned with the *peak* of the ongoing mu-alpha oscillations. We here hypothesize that such oscillatory phenomena emerge because rhythmic TMS is likely more effective at imposing entrainment when cortical activity is silenced or in inhibited states (e.g. at the peak of mu-alpha ongoing oscillation), hence in cycle phases with lower endogenous fluctuations, hence more sensitive to external frequency imposed entrainment. In contrast, a brain region hosting oscillatory reverberating activity will present higher resilience to externally imposed suprathreshold rhythmic inputs and become less sensitive to phase-resetting at the onset of a TMS pulse or burst, especially if the frequency of the former differs from the endogenous ongoing oscillation rhythm [67,68].

Accordingly, when active high intensity single pulse TMS and rhythmic TMS trials were sorted according to the phase (*peak* vs. trough) of the ongoing beta rhythm -most likely emerging from M1 in the somatosensory cortex- a larger and higher TMS beta-imposed oscillatory activity was observed when TMS was delivered at the *trough* of this ongoing rhythm. This outcome suggests that this specific phase of the cycle is associated with a brain state with less endogenous resistance, during which TMS may be more effective at phase-resetting, hence to effortlessly impose an external rhythm. The former effect could be speculatively interpreted as a state of beta 'pulsed facilitation', more easily enabling beta entrainment or beta rebound within sensorimotor systems for TMS pulses aligned to a specific phase of an ongoing rhythmic activity. However, since rhythmic TMS bursts and single pulse TMS at high intensity are likely subtended by different physiological mechanisms, further research, outside the scope of the present work, should explicitly confirm and investigate the basis of such an unexpected observation.

Prior work in rodents using electrical stimulation have shown that weak currents may selectively entrain alpha rhythms originating from the spiking of inhibitory interneurons[11]. Likewise, human neuroimaging and pharmacological studies have reported a causal role for alpha oscillations in increased local inhibitory tone and activity[69–73]. Prior studies have also unearthed weak (30V/m) and mild intensity (50V/m induced current density) TMS might be sufficient to entrain cortical alpha oscillatory activity and to modulate corticospinal excitability[22,23]. Albeit using lower levels of low intensity-TMS intensity (Li-TMS, 5-12V/m induced current density) and distinct stimulation frequency (20Hz), our study reported similar outcomes; notably an increase of alpha oscillatory activity for TMS pulses or bursts delivered during local inhibited states, such as the *trough* of mu-beta oscillations. This facilitatory effect induced at very weak TMS intensity was constrained to phase-specific conditions and elicited spectral power increases at an alpha frequency, a rhythm which has been linked to local inhibitory GABAergic neural activity[71] and thought to be primary originated in somatosensory cortex involving large feedforward inhibition between sensory and primary motor areas[74].

Prior studies[22,23] have suggested that TMS at low levels of intensity might entrain alpha activity. Extending this evidence, our study suggests for the first time that the modulatory effects of very weak TMS intensity here described are not fully frequency-dependent. This outcome is likely explained by the fact that stimulation of such a low magnitude might not be strong enough to impose an external rhythm. Instead, very weak intensity TMS may however facilitate the spontaneous increase or entrainment of endogenous activity, mostly affecting neural populations highly sensitive to perturbations. Importantly, within sensorimotor systems, inhibitory interneurons, classically involved in the generation of the alpha rhythm and known for possessing low firing threshold could be predominantly perturbed, which may explain and generate the phase-dependent alpha rhythm effects observed in our study[69,70,74]. Moreover, the fact that rhythmic TMS bursts at very weak-intensity synchronized activity in the alpha range whereas the same patterns delivered at high-intensity facilitated activity in the beta band, indicates a divergence of mechanisms at play by these two stimulation patterns, which solely differ (same stimulated region, with identical procedures and control conditions) by their intensity. On such basis, we here hypothesize that while high-intensity TMS might entrain frequency-specific oscillations, very weak-intensity TMS may amplify in the sensorimotor cortex spontaneous activity from highly sensitive intracortical neuronal populations -most probably inhibitory interneurons or low-threshold neuronal populations- in a brain-state dependent matter. Further studies exploring in single pulses and short rhythmic bursts a full gradual range of TMS intensities between what we here defined and used as very weak (Li-TMS) and conventional (Hi-TMS) stimulation will be needed to provide additional support to this notion.

Conclusions and future directions.

We here explored and found for the first-time evidence in humans that very-weak rhythmic TMS patterns at unprecedented low levels of intensity in humans (3%MSO, ~5-10V/m, ~6%rMT) facilitate via mu-beta phase-dependent effects, alpha activity, potentially associated with a local release of inhibitory neocortical activity. Importantly, differences in phase-dependent

modulatory effects of rhythmic TMS at very weak vs. conventional 'high intensity' suggest that these operate through different physiological mechanisms, limiting direct comparison of their respective impacts. Further studies assessing the *online* and *offline* influences of longer TMS bursts, repetitive TMS patterns and dose-response relationships between elicited neuronal oscillatory activity and E-field impact will be needed to better pinpoint the potential of very weak-intensity TMS.

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Competing interests

The authors declare no competing financial interests.

Data and Code availability

The datasets produced and/or analyzed during the current study are not publicly accessible due to institutional regulations. However, they can be obtained from the corresponding and/or first author upon reasonable request. The codes for data analysis are accessible at the permanent link: https://github.com/XavierCorominas/Neural-signatures-of-weak-magnetic-stimulation.

Authorship contributions

XC-T and AV-C conceived and designed the study with conceptual input by MB, SC, CG, SM, RS, AL and MTC. XC-T and MB conducted the experiments and collected the data. XC-T assisted by TM, MB and AV-C, analyzed the data. XC-T, MB and AV-C prepared the first versions of the manuscript. All remaining authors contributed to critically discuss and read prior versions of this project and approved the final version of the manuscript.

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Supplementary information

Supplementary Materials, with 5 figures and 2 information sets accompanies this main text.

FIGURES

Figure 1. TMS-EEG data analysis pipeline employed in our study. MRI-Neuronavigated TMS-EEG was used to characterize the *online* impact (i.e., during stimulation periods) of *Single Pulse* and *Rhythmic* (20Hz frequency) TMS patterns on the left M1 cortex of our cohort of healthy participants. To this end, EEG data was pre-processed using a pipeline able to identify and subtract artifactual signals. In parallel, individualized tetrahedral finite element head models (FEM) reconstructed from T1 MRI volumes for each participant were used to estimate the E-field distribution, elicited by cortical currents delivered during experimental sessions to the left M1 cortex hotspot for the right First Dorsal Interosseous (FDI) hand muscle. This head model served to estimate the magnetic field strength and to delineate for each participant a specific region of interest (ROI) defined as the 80^{th} percentile of the predicted E-field strength. Minimizing mass conduction and multisensory afferent signal components, a spatial filtering pipeline encompassing the targeted regions of each specific participant was applied to reconstruct TMS modulated EEG sources and extract EEG activity signatures elicited by stimulation. Finally, analyses in the time-, frequency- and phase-domains were conducted to explore respectively: TMS evoked potentials (TEP), TMS spectral perturbations (ERSP), TMS inter-trial phase synchronization (ITPC) and oscillatory phase-dependent (bound to brain state) modulatory effects on TEP amplitude and ERSP power.

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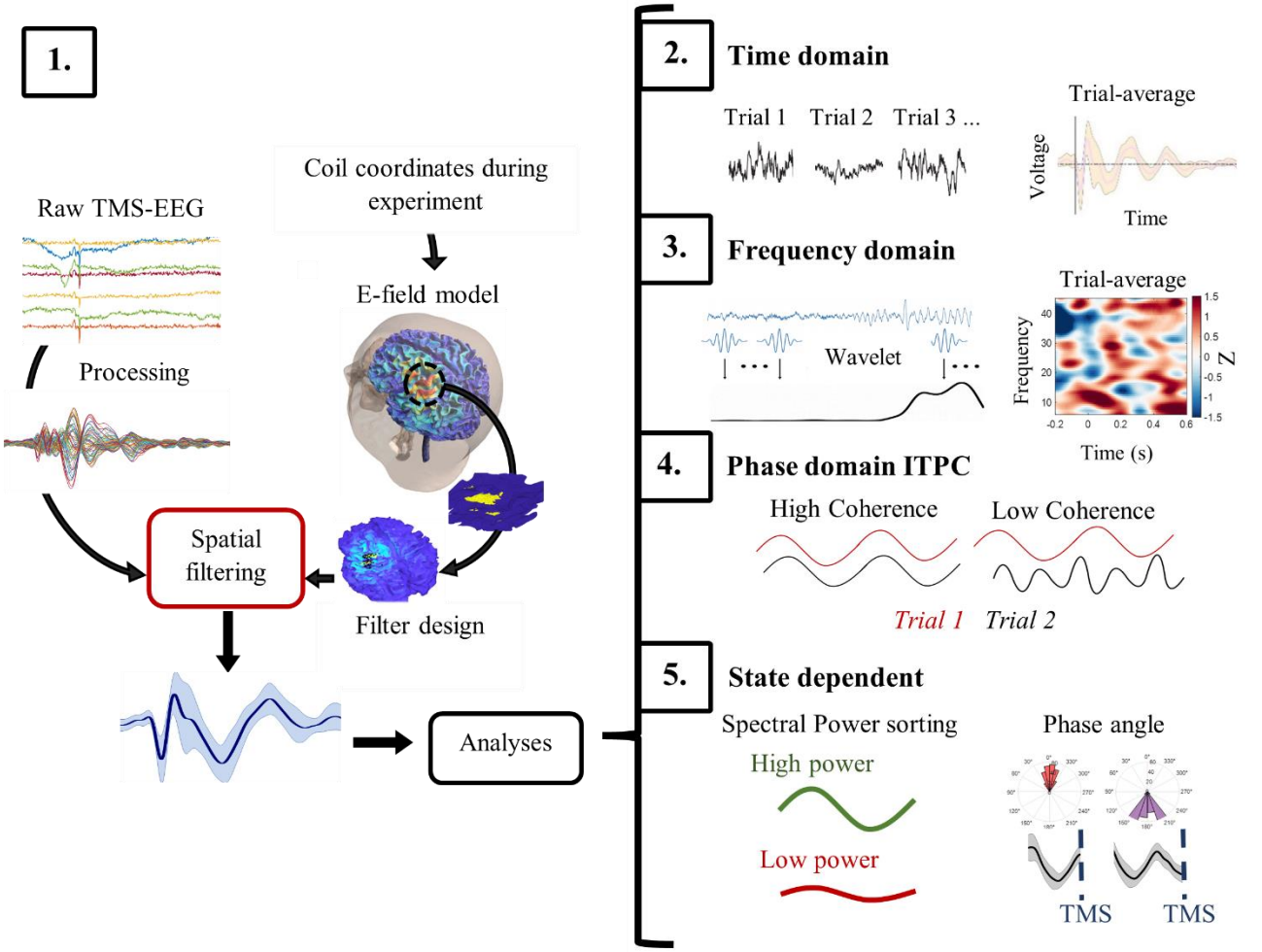

1.
Raw TMS-EEG
Processing
Coil coordinates during experiment
E-field model
Filter design
Spatial filtering
Analyses
2.
Time domain
Trial 1
Trial 2
Trial 3 ...
Trial-average
Voltage
Time
3.
Frequency domain
Wavelet
Trial-average
Frequency
Time (s)
4.
Phase domain ITPC
High Coherence
Low Coherence
Trial 1
Trial 2
5.
State dependent
Spectral Power sorting
High power
Low power
Phase angle
TMS
TMS

Figure 2. Effects of high and very weak-intensity single pulse TMS on Transcranial Evoked potentials (TEP). All trials have been averaged across participants and grouped by specific TMS condition at high (Hi-TMS, ~113V/m, 60%MSO, ~77% rMT), and at very weak-intensity (Li-TMS, ~6 V/m, 3%MSO, ~6% rMT), here referred to as Hi-TMS and Li-TMS, respectively. The group mean and standard deviation (Mean±SD) values per each condition are indicated with a thick centered line and a thin background line, respectively. Vertical straight continuous lines signal the onset time of each individual TMS pulse within a burst. Vertical gray bars indicate statistically significant EEG time-frequency clusters ($p<0.05$, two-sided) in the time time domain between conditions. Hi-TMS: high-intensity TMS (60%MSO, ~113V/m, ~77% rMT), Li-TMS: very weak-intensity (3%MSO, ~6V/m, ~6% rMT). rMT: resting Motor Threshold; MSO: Maximal Stimulator Output.

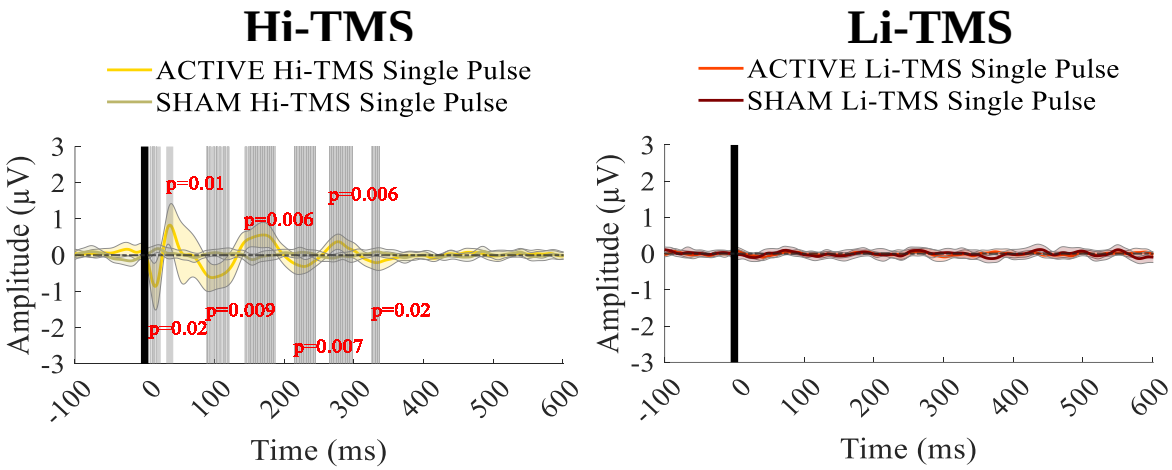


Figure 3. Effects of high and very weak-intensity TMS on Event-Related Spectral Perturbation (ERSP) measures. Time-frequency series displaying Event-Related Spectral Perturbations (ERSP) across experimental conditions. All trials have been averaged across participants and grouped by specific TMS conditions (*Single Pulse TMS* and *Rhythmic TMS* at high and very weak-intensity stimulation, referred as Hi-TMS and Li-TMS respectively). ERSP data displayed in the figure correspond to Z-transformed group grand averages organized for each TMS condition. Comparisons between Active vs. Sham independently for *Single Pulse* and *Rhythmic* TMS and for high-intensity (Hi-TMS) and very weak-intensity (Li-TMS) TMS pulses or bursts are presented in the figure. Black outlines indicate statistically significant EEG clusters in the time and frequency domains ($p<0.05$, two-sided). Hi-TMS: High-Intensity TMS (60%MSO, ~113V/m, 77% rMT), Li-TMS: very weak-intensity (3%MSO, ~6V/m, 6% rMT). rMT: resting Motor Threshold; MSO: Maximal Stimulator output.

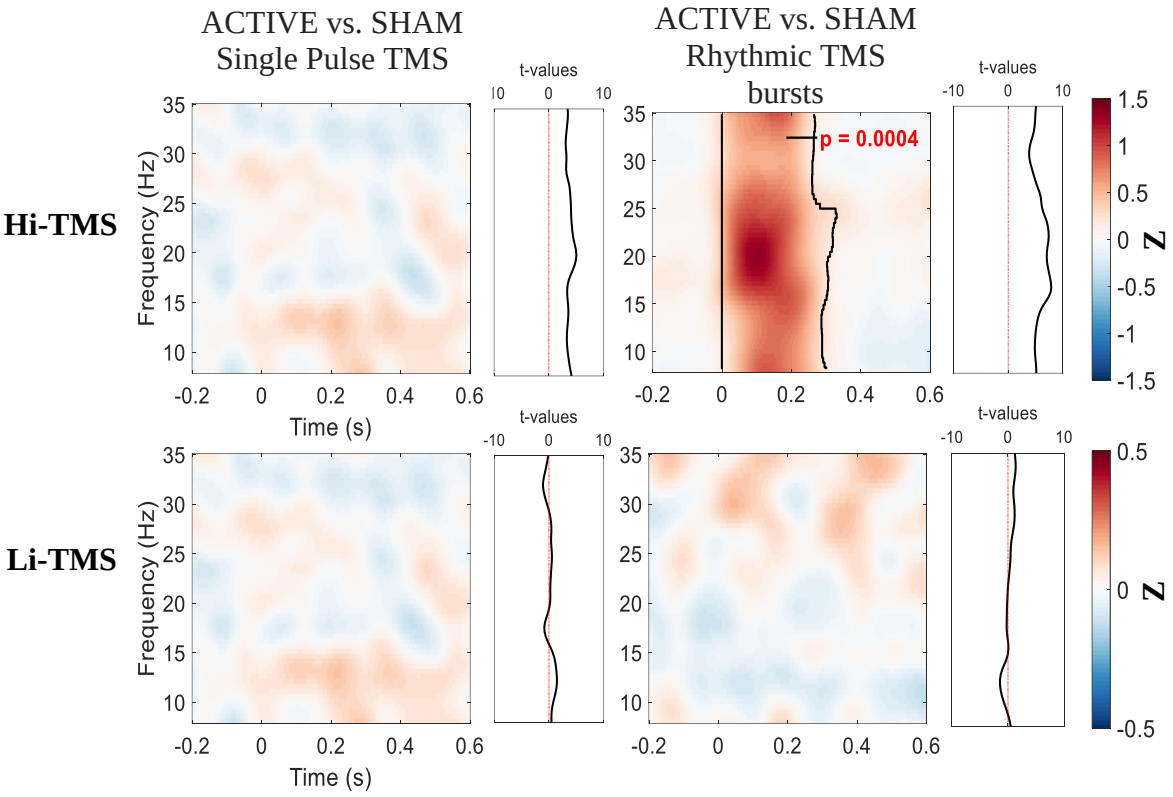


Figure 4. Effects of high and very weak-intensity TMS on Intertrial Phase Coherence (ITPC) measures. A. Time-frequency series displaying Intertrial Phase Coherence (ITPC) within the motor cortex region across experimental conditions. Displayed ITPC data correspond to grand averages across participants grouped by each TMS conditions tested in our study (*Single Pulse TMS* and *Rhythmic TMS* at high and very weak-intensity stimulation, Hi-TMS and Li-TMS respectively). Comparisons are shown for Active vs Sham for *Single Pulse TMS* and *Rhythmic TMS* (after subtracting their respective Sham conditions) for high-intensity (Hi-TMS) and very weak-intensity (Li-TMS) stimulation TMS pulses or bursts. Black outlines indicate statistically significant EEG clusters in the time and frequency domains ($p<0.05$, two-sided). **B.** Total number of genuine oscillatory entrainment episodes per subject and conditions after aperiodic 1/f parametrization employing the fBOSC algorithm. Conditions were compared across an equal number of random selected trials (612xCondition) centered around the stimulated frequency (8-35Hz) and TMS events (-100ms 400ms).

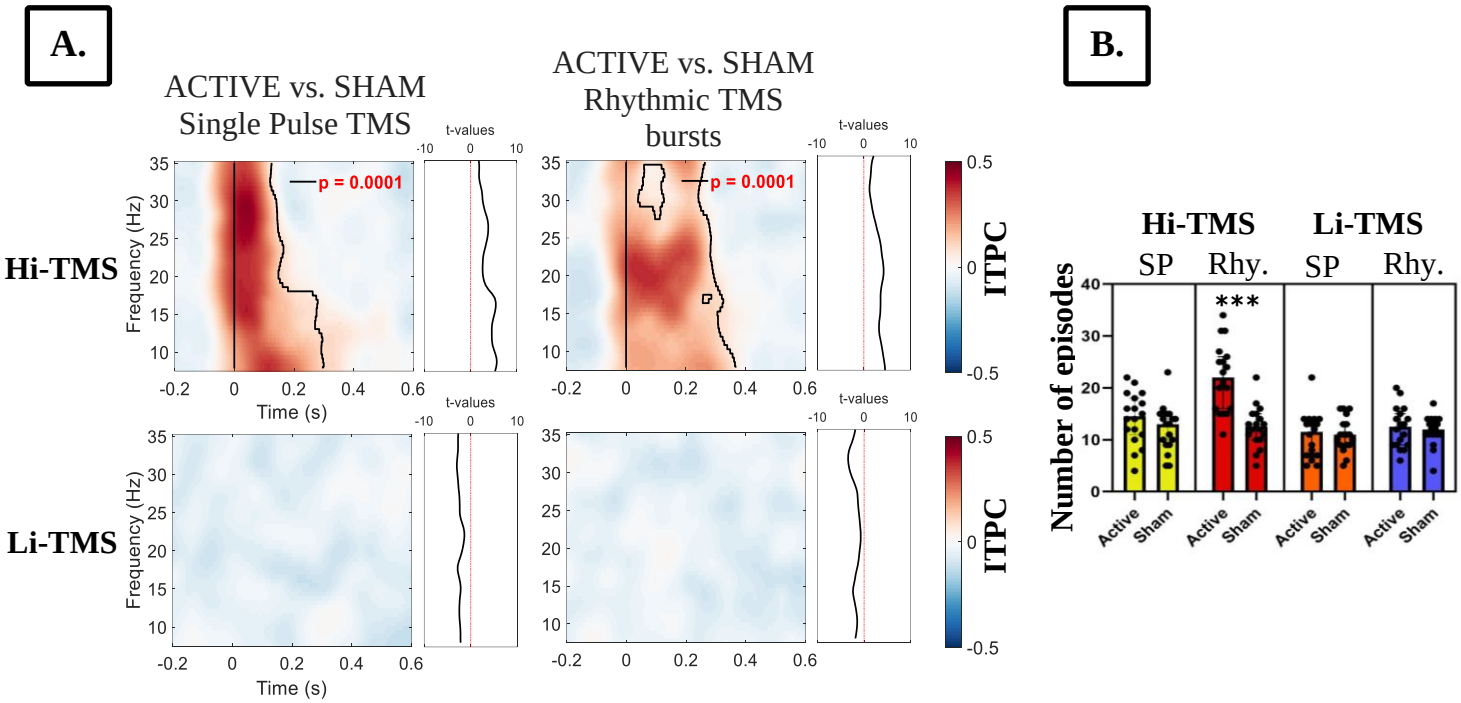


Figure 5. Mu-alpha phase-dependent TMS effects on evoked potentials and Event-Related Spectral Perturbation (ERSP) estimates . TMS evoked potentials and time-frequency series of Event-Related Spectral Perturbation (ERSP) measurements sorted according to the instantaneous pre-TMS onset phase (-5ms to -2ms) of the mu-alpha rhythm (8-13Hz). More specifically, active TMS trials (exceeding +50% of total oscillatory power across all active trials, sham subtracted) at the *peak* or the *trough* of the mu-alpha rhythm at the time of 1st TMS pulse onset were identified and selected for final analysis. Time-series for active TMS conditions contrasting *peak* vs *trough* sorted trials are displayed in the figure. Red lines and clusters of EEG plots indicate increased evoked amplitude for TEPs (left column) or oscillatory power increase for the ERSPs (right column) when TMS pulses were delivered around the *peak* of the ongoing mu-alpha oscillation. Blue lines and clusters of EEG sensors indicate increased evoked activity for TEP amplitude (left column) or oscillatory power increase from ERSPs recordings (right column) when TMS pulses were delivered around the *trough* of the ongoing mu-alpha oscillation. In TEP plots, dark black vertical lines denote TMS pulses, while gray vertical lines indicate statistically significant differences ($p<0.025$, two-sided) between conditions in the time domain. For time-frequency ERSPs representations, black outlines indicate statistically significant clusters between conditions in the time and frequency domains ($p<0.05$, two-sided). Hi-TMS: High-Intensity TMS (60%MSO, ~113V/m, ~77% rMT), Li-TMS: very weak-intensity (3%MSO, ~6V/m, ~6%rMT). rMT: resting Motor Threshold; MSO: Maximal Stimulator output.

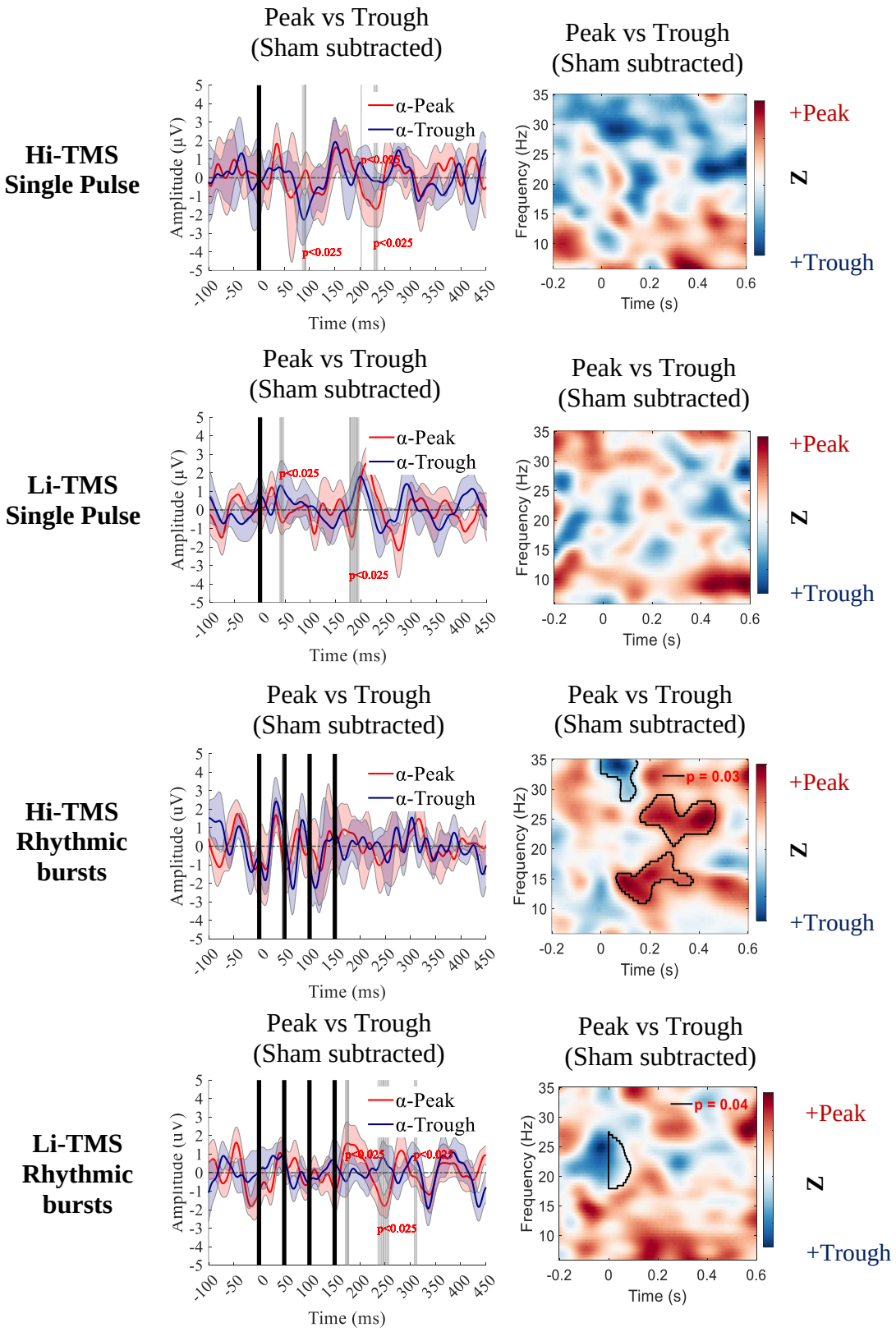
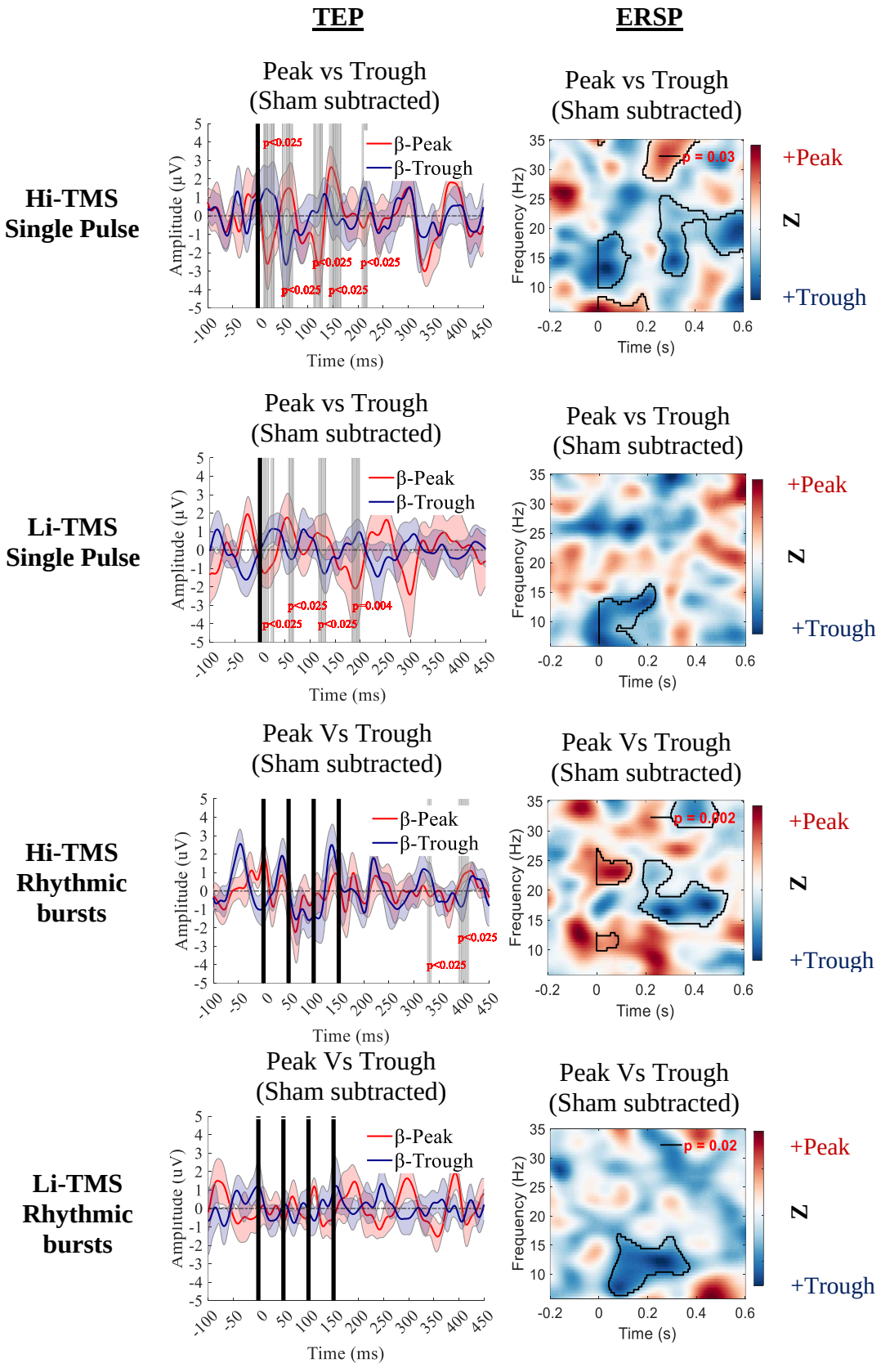
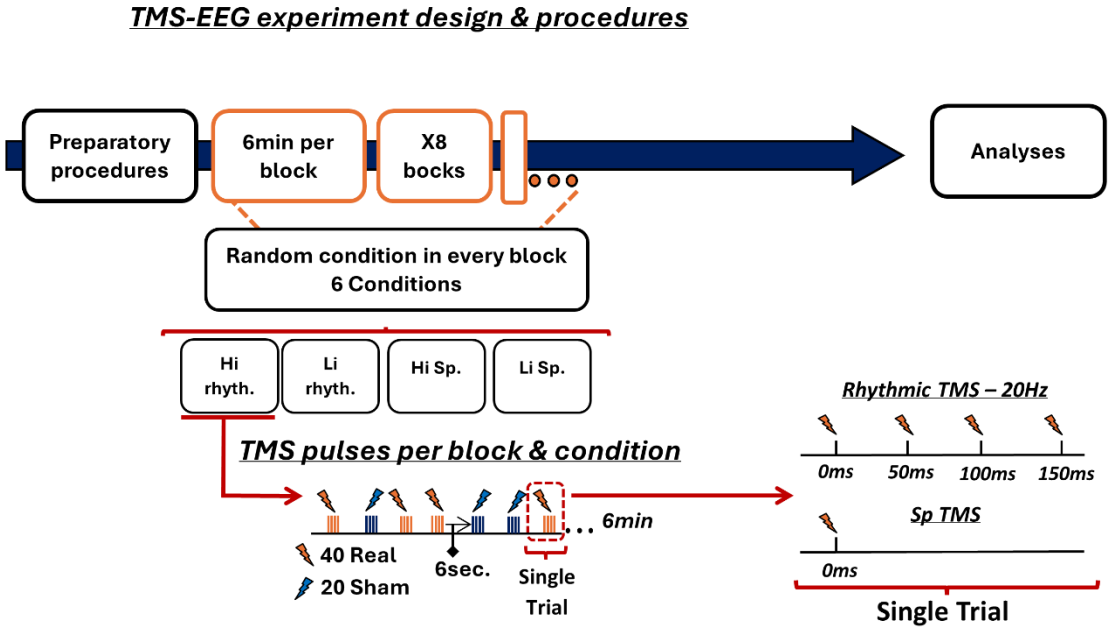

TEP
ERSP
Hi-TMS Single Pulse
Li-TMS Single Pulse
Hi-TMS Rhythmic bursts
Li-TMS Rhythmic bursts
Peak vs Trough (Sham subtracted)
α-Peak
α-Trough
Amplitude (μV)
Amplitude (uV)
Time (ms)
p<0.025
Frequency (Hz)
Time (s)
p = 0.03
p = 0.04
+Peak
Z
+Trough

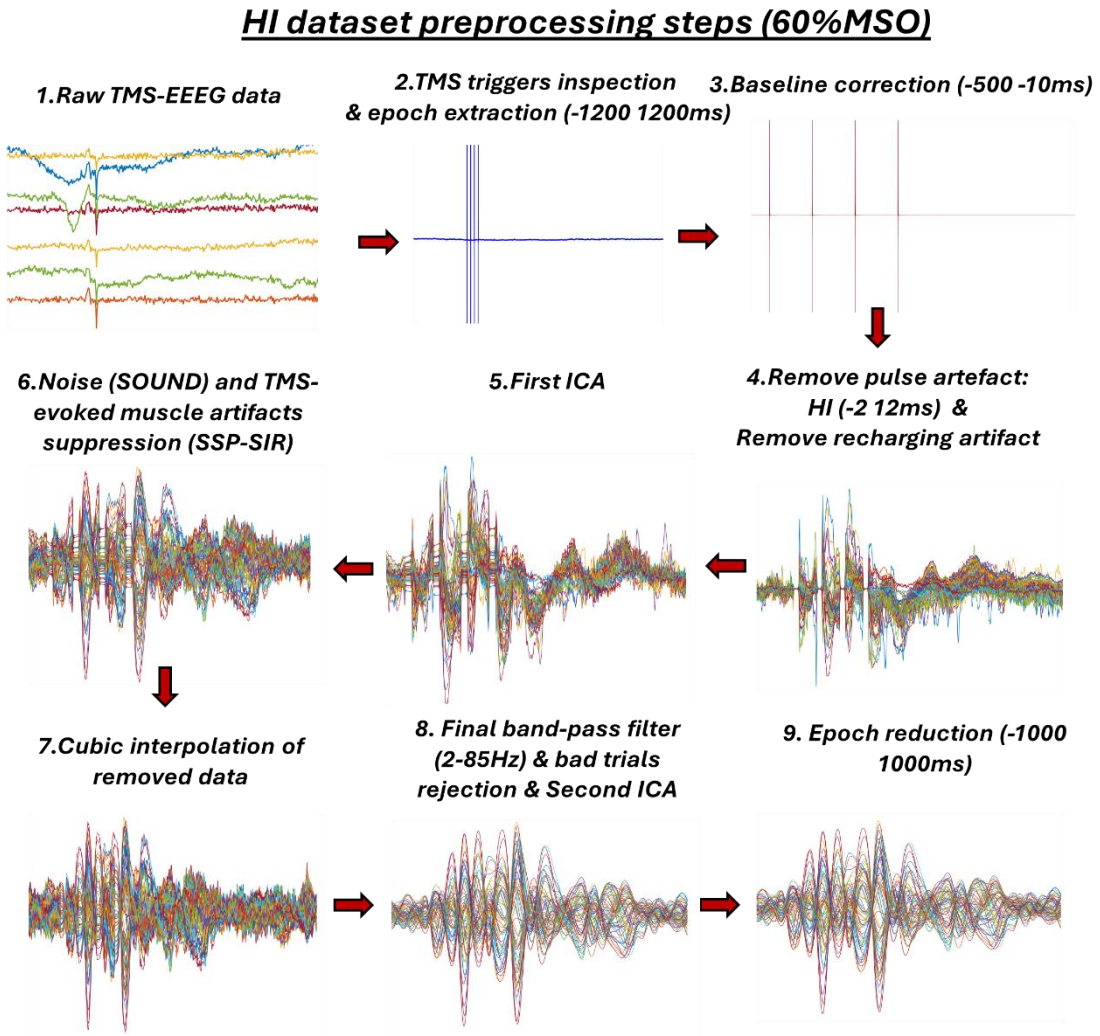
Figure 6. Mu-beta Phase-dependent TMS effects on evoked potentials and Event-Related Spectral Perturbation (ERSP) measurements. TMS evoked potentials and time-frequency series sorted according to the instantaneous pre-TMS phase (-5ms to -2ms) of the mu-beta (14-30Hz) rhythm. Active TMS trials (exceeding +50% of total oscillatory power across all active trials, after subtraction of their associated sham conditions) at the *peak* or the *trough* of the mu-beta rhythm at the time of the 1st TMS pulse onset were identified and selected for final analysis. Time-series for active TMS conditions contrasting *peak* vs *trough* sorted trials are displayed in the figure. Red lines and clusters of EEG activity indicate increased evoked amplitude for TEPs (left column) or oscillatory power increases in ERSPs (right column) when TMS pulses were delivered around the *peak* of the ongoing mu-beta oscillation. Blue lines and EEG clusters plots indicate increased evoked activity for TEPs (left column) or oscillatory power increase in ERSPs (right column) when TMS pulses were delivered around the *trough* of the ongoing mu-beta oscillation. In TEPs plots, dark black vertical lines denote TMS pulses, while gray vertical lines indicate statistically significant differences ($p<0.025$, two-sided). For time-frequency ERSPs plots, black outlines indicate statistically significant clusters ($p<0.05$, two-sided). Hi-TMS: High-Intensity TMS (60%MSO, ~113V/m, 77% rMT), Li-TMS: very weak-intensity (3%MSO, ~6V/m, 6% rMT). rMT: resting Motor Threshold;. MSO: Maximal Stimulator output.


TEP
ERSP
Hi-TMS Single Pulse
Li-TMS Single Pulse
Hi-TMS Rhythmic bursts
Li-TMS Rhythmic bursts
Peak vs Trough (Sham subtracted)
Peak Vs Trough (Sham subtracted)
β-Peak
β-Trough
Amplitude (µV)
Amplitude (uV)
Time (ms)
Frequency (Hz)
Time (s)
p<0.025
p=0.004
p = 0.03
p = 0.002
p = 0.02
+Peak
Z
+Trough

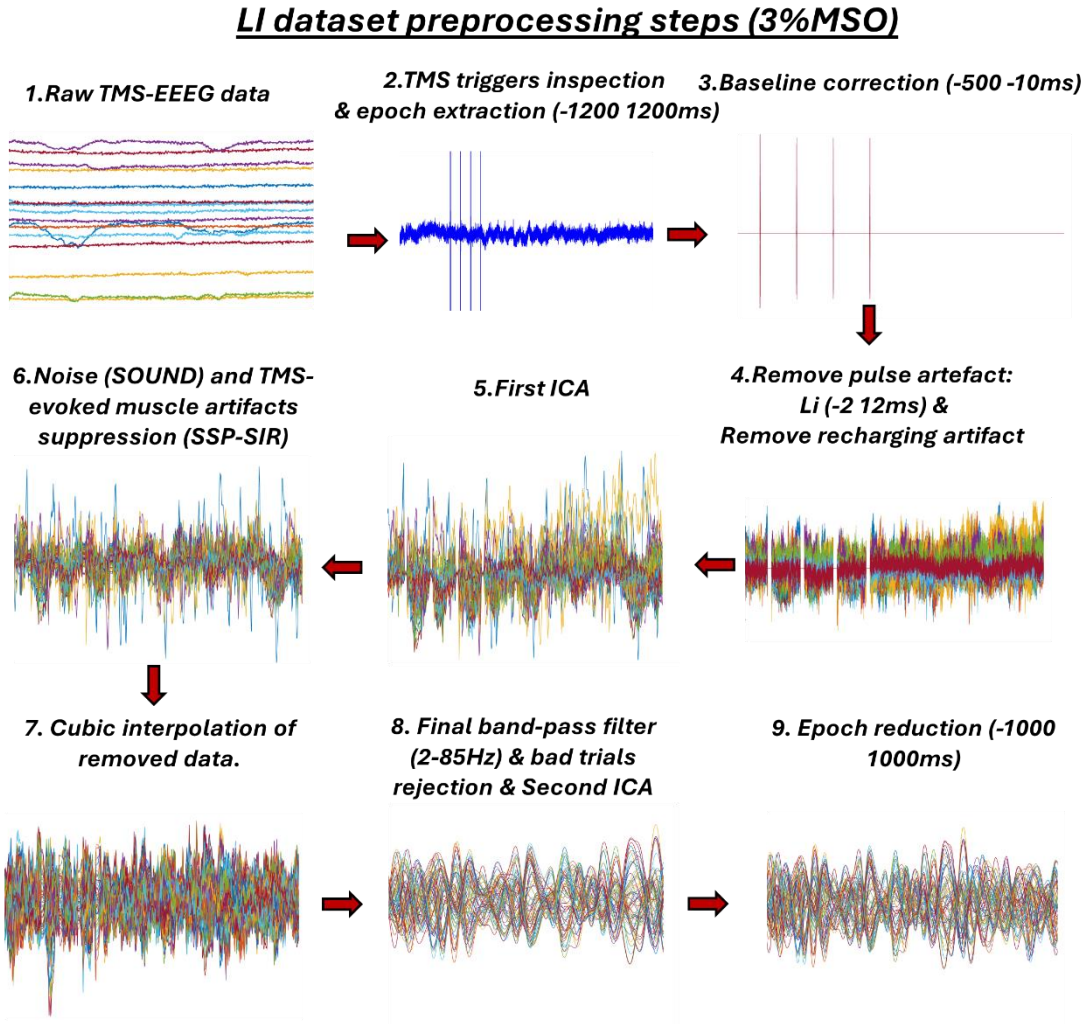
Supplementary Figure 1. Schematic illustration of experimental design and procedures employed in our study. A series of preparatory procedures described in detail in the General Procedures and TMS protocols sections of our manuscript preceded the main experiment. The experiment contained a total of experimental conditions: ACTIVE/SHAM Single Pulse TMS (referred to in figures as SP TMS) and ACTIVE/SHAM rhythmic TMS (at 20Hz) stimulation all delivered on the left primary motor cortex, hotspot of the right First Dorsal Interosseus (FDI) hand muscle at conventional TMS intensity (Hi-TMS) or at very weak intensity (Li-TMS). Every condition was repeated twice totalling 8 blocks per participant. SHAM stimulation was embedded randomly within each ACTIVE TMS block; therefore a total of 40 ACTIVE pulses/bursts and 20 SHAM embedded pulses were delivered on each block.



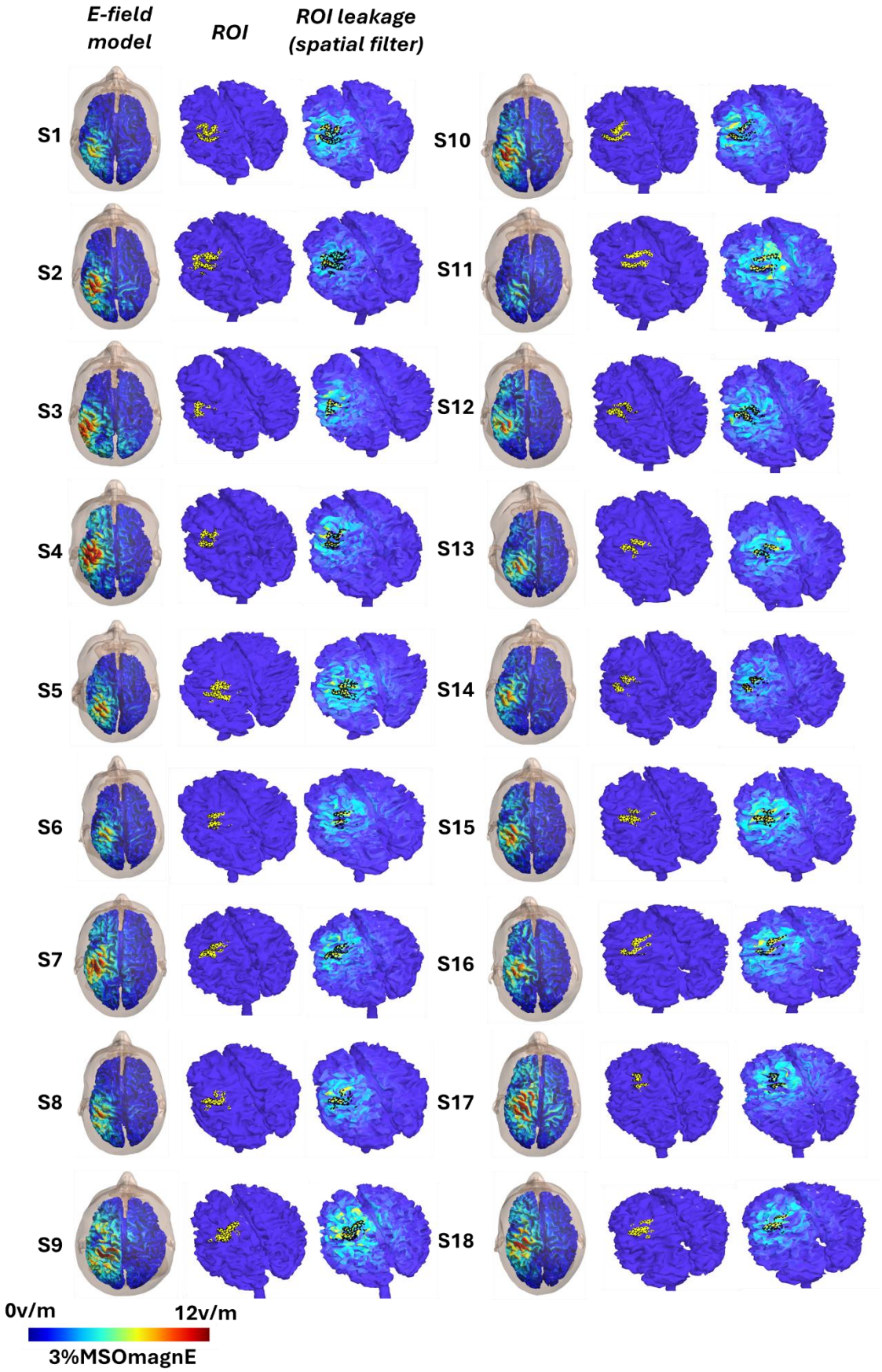
Supplementary Figure 2.1 Example of TMS-EEG pre-processing and cleaning procedure applied to the rhythmic high-intensity (60%MS0) TMS dataset from a representative participant. Full details can be found in the TMS-EEG pre-processing and artifact removal subsection within the methods section of the main manuscript.



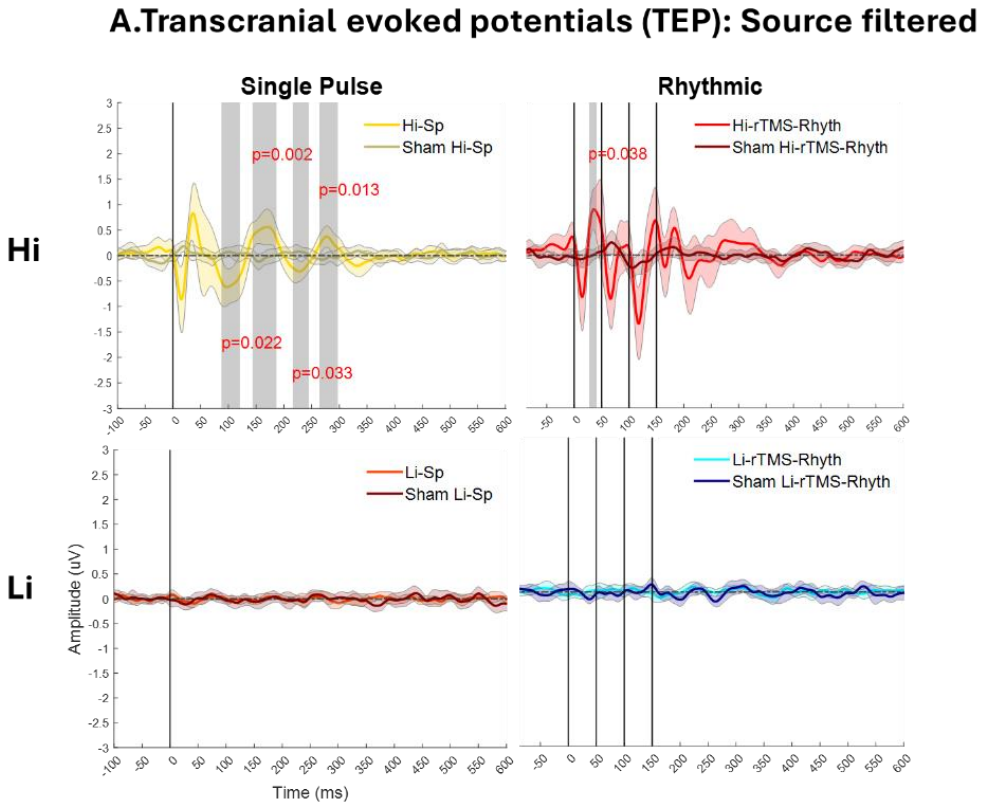
Supplementary Figure 2.2 Example of TMS-EEG pre-processing and cleaning procedure applied to the rhythmic low-intensity (3%MS0) TMS dataset of a representative participant. Full details can be found in the TMS-EEG preprocessing and artifact removal subsection within the methods section included in the main manuscript.

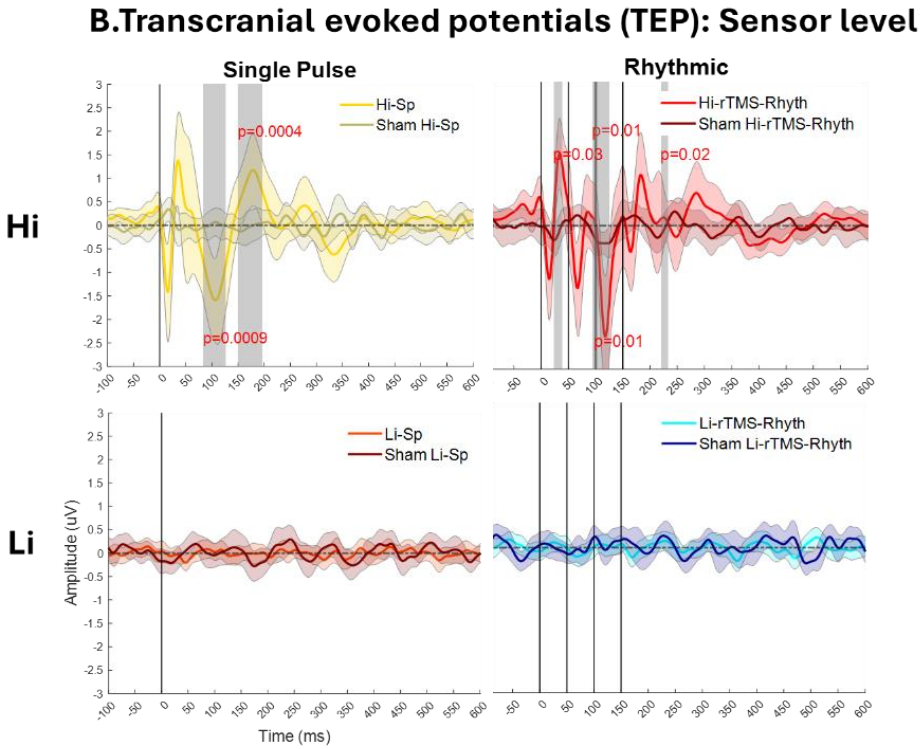


Supplementary Figure 3. MRI based E-field spread simulations defining the boundaries of the estimated region of interest (ROI) primarily impacted by the TMS pulses or bursts. The ROI was estimated considering the 80th percentile of the maximum peak strength and spatial filter crosstalk leakage sensitivity levels specific for each individual participant, from subject 1 (S1) to subject 18 (S18). For visualization purposes, individual E-field values have been normalized from 0V/m (minimum) to 12V/m (maximum). Individual E-field simulations were computed by reconstructing each participant's structural head model with SIMNIBS4.0. Note that all other sources non-highlighted in the ROI leakage figure were minimized by the spatial filtering procedure employed in this study. Accordingly,, crosstalk leakage figures can also be interpreted as a source-individualized reconstruction of the original location of the extracted signal for each participant.


E-field model
ROI
ROI leakage (spatial filter)
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
S17
S18
0v/m
12v/m
3%MSOmagnE

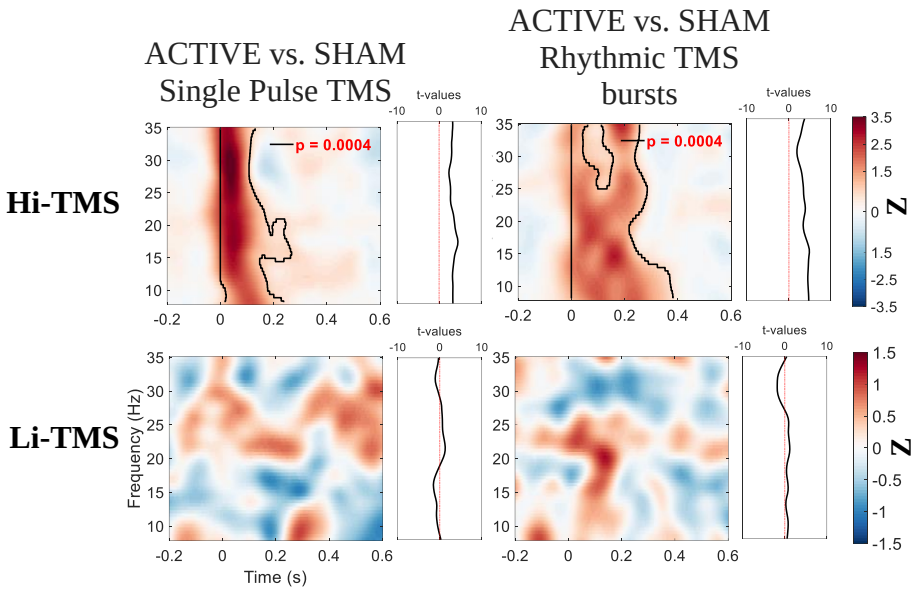
Supplementary Figure 4. Time-course of Transcranial Evoked potentials (TEPs) evoked by the single pulse TMS conditions tested in our study. All trials have been averaged across participants and grouped by specific TMS conditions at high (Hi-TMS), and at very weak-intensity (Li-TMS) stimulation. The group mean and standard deviation (±SD) values for each condition are indicated with thick and a thin line profiles, respectively. Vertical straight solid lines signal the onset time of each individual TMS pulse. Vertical grey-shaded areas indicate statistically significant EEG clusters in the time and frequency domains (Montecarlo cluster based permutation statistics, cluster alpha $p<0.05$, Montecarlo alpha $p<0.05$, two-sided) across conditions. Hi-TMS: high-intensity TMS (60%MSO, ~113V/m), Li-TMS: low-intensity (3%MSO, ~6V/m). **A.** Spatially filtered TEP for all conditions. **B.** TEP responses of non-spatially filtered signals (original EEG signals excluding the application of E-field-informed spatial filtering procedures implemented in our final analyses) at the group level from a cluster of electrodes overlying the stimulated left primary motor (M1). The EEG electrodes specifically selected for the analyses were: C3, CP1, C1 and CP3. For this figure, TEP analyses and statistical comparisons were applied as described in the main manuscript but excluding spatial filtering preprocessing step in sensor level TEP time courses.


A.Transcranial evoked potentials (TEP): Source filtered
Single Pulse
Rhythmic
Hi
Li
Hi-Sp
Sham Hi-Sp
Hi-rTMS-Rhyth
Sham Hi-rTMS-Rhyth
Li-Sp
Sham Li-Sp
Li-rTMS-Rhyth
Sham Li-rTMS-Rhyth
p=0.002
p=0.013
p=0.022
p=0.033
p=0.038
Amplitude (uV)
Time (ms)

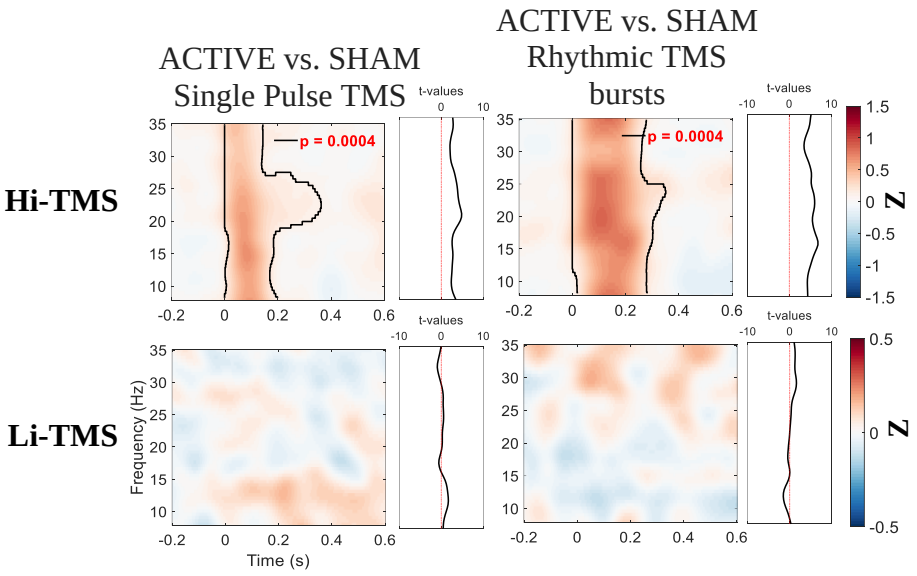

B.Transcranial evoked potentials (TEP): Sensor level
Single Pulse
Rhythmic
Hi
Li
Hi-Sp
Sham Hi-Sp
Hi-rTMS-Rhyth
Sham Hi-rTMS-Rhyth
Li-Sp
Sham Li-Sp
Li-rTMS-Rhyth
Sham Li-rTMS-Rhyth
p=0.0004
p=0.0009
p=0.01
p=0.03
p=0.02
p=0.01
Amplitude (uV)
Time (ms)

Supplementary Figure 5. Evoked and induced oscillatory responses from the spatially filtered and source reconstructed data. Upper panels represent Evoked oscillatory responses (EOR) while lower panels represent induced oscillatory responses (IOR). EOR represents the averaged trial-to-trial time-frequency activity of the time- and phase-locked activity, where evoked potentials (TEP) are first computed and averaged across trials to be then spectrally deconstructed. IOR represents the averaged trial-to-trial time-frequency activity of the non-time- nor phase-locked activity, where EOR are subtracted from the total oscillatory responses (TOR: time-frequency is performed for each single trial and then averaged). Following EOR and IOR computation, data was treated following ERSP analysis procedures (z-transformed and baseline corrected) and cluster statistics were applied.

Evoked oscillatory responses



Induced oscillatory responses



Supplementary Materials.

Supplementary Materials 1

For high-intensity blocks, TMS intensity was set at a fixed level of 60% of the maximum stimulator output (MSO). Importantly, we verified that this level of intensity warranted the full recharge of the TMS stimulation boosters and an accurate and consistent delivery of TMS 4 pulse bursts avoiding intensity inaccuracy or missing pulses during rhythmic TMS patterns. Prior evidence has demonstrated that an intensity of ~80% of the resting motor threshold (rMT) is sufficient to induce measurable scalp EEG responses in absence of peripheral MEPs, hence minimizing the interference of muscle artifacts[1,2] or proprioceptive afferent feedback. Importantly, our post-hoc analyses (see Supplementary Table 1) confirmed that the fixed intensity of 60% MSO used in our study matched the ~80% rMT values for the cohort (mean rMT=77%MSO; 80% of MT=61%MSO).

For low-intensity TMS blocks, we aimed to induce on the M1 surface voltage density levels similar to those applied in rat studies published with the participation of our lab and accounting for currents of ~0.05-0.1V/m. Given obvious differences between rodent and human scalp thickness, we carried out series of MRI-based tetrahedral Finite Element Model (FEM) simulations of TMS M1 current density distribution on a standard average 152 MNI brain (see FEM simulations section of the main manuscript) at different TMS intensities with this same equipment (Magstim, Rapid2, 70 mm double coil). On such basis, our quest predicted that the intensity matching most closely the one delivered in prior studies with a custom-made micro-TMS stimulator device (~0.05-0.1V/m) would need to be of ~1% MSO, 1.14 dI/dtmax [A/µs]). An in-house induction coil probe attached to a digital oscilloscope (Tektronic 6485, USA), served to estimate the lowest magnetic field output (1-100% MSO) that could be consistently delivered with our human

use certified rTMS equipment (Magstim Rapid^2) and coil (70 mm coil) by measuring on its center the strength of the evoked magnetic field peak voltage at increasing TMS intensities (from 1-10%MSO). We determined that the minimal intensity offering consistent regular pulse output was 3% MSO (3.44 dI/dtmax [A/µs]). Accordingly, 3% MSO (predicted to achieve on M1 a current density of ~5-12V/m depending on interindividual head volume differences across subjects) was employed in all our participants as the closest proxy of very weak intensity stimulation (compared to the one tested in rodents).

Supplementary Materials 2

The meshes contained 10 different volumes (0.6mm isotropic resolution) segmented from the following tissues: white matter (WM), grey matter (GM), cerebrospinal fluid (CSF), bone, scalp, eyes, compact bone (CB), spongy bone (SB), blood vessels and muscles. The following isotropic conductivity values were assigned: 0.126 S/m (WM), 0.275 S/m (GM), 1.654 S/m (CSF), 0.01 S/m bone, 0.16 S/m scalp, 0.5 S/m eyes, 0.008 S/m CB, 0.025 S/m SB, 0.6 S/m blood vessels, and 0.46 S/m muscles. Tissue segmentation was performed automatically relying on CAT12 and SPM12 and manually corrected when required after careful visual inspection. Electrical fields were estimated assuming a quasi-static regime, according to

$$E = -\partial A/\partial t - \nabla\varphi$$

where E is the electric field vector and φ the electric potential. Models were computed with a Magstim figure-of-eight 70 mm coil (D70) powered by a MagstimRapid^2 (114.7 dI/dtmax [A/µs] = 100%MSO). Since the relationship between A/µs and %MSO is linear, TMS coil rate of change (dI/dt) was set to 3.4A/µs for the low intensity TMS simulations (Li-TMS, 3% MSO) and to 68.8A/µs for conventional high-intensity models (Hi-TMS,

60% MSO). TMS coils were spatially placed according to the neuronavigation actual positions (Brainsight, Rogue Solutions) used during the experimental session and manually corrected when necessary, maintaining a 7mm coil-to-scalp distance to simulate the presence of active EEG electrodes. Biophysical simulations of current distribution in our study served two purposes. Firstly, they allowed us to estimate for each individual participant the peak current generated by Li-TMS (3%MSO) and Hi-TMS (60%MSO) intensities employed on the left M1 cortical target. Secondly, they served to construct individual spatial filters to process EEG data at the source level.

Supplementary References

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[3] Opitz A, Windhoff M, Heidemann RM, Turner R, Thielscher A. How the brain tissue shapes the electric field induced by transcranial magnetic stimulation. Neuroimage 2011;58:849–59. https://doi.org/https://doi.org/10.1016/j.neuroimage.2011.06.069.

Supplementary Tables

Supplementary Table 1. Demographic information on the included cohort of n=18 healthy participants. Sex (M: male,. F: Female), Age (in years of age), Resting Motor Threshold value (in % of the Maximal Stimulator Output) and individual values of the predicted 99th percentile E-field impact extracted from individual E-field simulations (SIMNIBS 4.0) on the cortical gray matter.

SUBJECT ID	SEX	AGE	RESTING MOTOR THRESHOLD (%MSO)	3%MSO E-field impact V/m (99th percentile)	60%MSO E-field impact V/m(99th percentile)
01	F	26	76	5.9	112.8
02	F	25	68	7.0	124.2
03	M	35	82	8.0	105.7
04	M	27	84	9.2	125.3
05	M	49	77	6.6	134.0
06	F	48	72	5.1	102.1
07	F	25	75	7.7	114.1
08	F	25	70	5.6	115.8
09	M	23	95	7.6	87.5
10	F	23	70	7.3	129.0
11	M	23	78	3.1	71.0
12	F	24	85	5.9	117.6
13	F	25	87	5.6	111.9
14	M	27	70	5.1	102.9
15	M	27	72	6.0	110.1
16	F	28	74	6.0	119.8
17	M	27	73	7.9	108.0
18	F	24	83	7.3	146.9
Group average	**10F/8M**	**28.2**	**77.2**	**6.4**	**113.9**

4.2.2 Manuscript 4: Entrainment with subthreshold non-invasive transcranial stimulation of the human primary motor cortex : A TMS-EEG pilot study using from very-weak to near-threshold intensity pulses

Entrainment with subthreshold non-invasive transcranial stimulation of the human primary motor cortex : A TMS-EEG pilot study using from very-weak to near-threshold intensity pulses

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ABSTRACT

Repetitive Transcranial magnetic stimulation (TMS) at near-threshold intensity has been employed for several decades to modulate neural activity in the context of experimental and clinical applications. Despite evidence informing on signs of online and offline modulation in rodents of the so-called 'weak' to 'very weak' TMS intensities, human studies in this area remain scarce. More specifically, the response of the human primary motor cortex (M1) to different levels of subthreshold TMS intensities likely to operate on different neuronal subsystems according to their individual activation thresholds remains poorly explored. We here investigated in a cohort of n=4 healthy participants, the EEG signature of short beta (20 Hz) TMS bursts delivered to the left M1 cortex while we continuously monitored electroencephalographic activity (EEG). More specifically, bursts at different intensities allowed us to establish a dose-response (input-output) function between five subthreshold TMS intensity levels indexed individually (10, 30, 50, 70, and 90% of the resting motor threshold, rMT) and cortical evoked outputs. As expected, rhythmic bursts at intensities neighboring the motor threshold (70 and 90% rMT) elicited consistent beta spectral perturbations and showed signs of phase synchronization. More surprisingly, low-intensity TMS (at 30% rMT) elicited episodic beta desynchronization. Considering prior evidence combining TMS and pharmacological perturbations, we here hypothesize a selective entrainment of inhibitory rhythms enabled specifically by low TMS intensities and different mechanisms of action than TMS at conventional stimulation. This evidence adds to recent reports on the modulatory power of very weak intensity TMS on neural systems in rodents and humans, which if confirmed might prove crucial for developing multi-channel and low consumption stimulation devices and novel applications.

Keywords: Noninvasive brain stimulation, Neuromodulation, Weak intensity and Low-intensity transcranial magnetic stimulation, Primary motor cortex, Motor systems, Oscillation entrainment, Synchrony, Brain-state dependent stimulation.

HIGHLIGHTS

1. We compiled dose response (input-output) recruitment curves of beta entrainment by short bursts of subthreshold TMS intensity under EEG monitoring.
2. Subthreshold threshold beta TMS bursts, at 50 and 70% of the resting motor threshold delivered to M1 elicited frequency-specific perturbations and local synchronization.
3. Mimicking previously reported GABAergic-release signatures, low-intensity TMS indexed to 30% of the resting motor threshold induced episodic beta desynchronization.

INTRODUCTION

For more than three decades now, Transcranial Magnetic Stimulation (TMS) has been employed as a tool to either suppress, boost, interfere or modulate regional and network correlates of brain activity[1,2]. Despite an increasingly prominent role in causal brain mapping studies and for the treatment of neuropsychiatric diseases, our comprehension of the TMS neural mechanisms of action[3] and the relation of such with dose-dependent modulatory response remains incomplete. This is particularly notorious when this technique is used in frequency-specific repetitive patterns at parameters of intensity[4] well-below the so-called 'resting motor threshold' (rMT). Along with the different factors with bearing on individual dose-response effects (notably, the number and periodicity of TMS sessions, stimulation parameters such as intensity and frequency, combined and interacting with anatomical and physiological features uniquely associated to each individual), the importance of TMS intensity and its ability to impact primary activated structures or influence net excitatory/inhibitory output has been generally underrated.

Magnetic pulse intensity is commonly indexed to a threshold level able to elicit measurable physiological outputs such as motor-evoked potentials (MEP) with peak cortical strengths of ~100-150V/m[5–8]. Nonetheless, much weaker field strengths (~0.5V/m) of Transcranial Electric Stimulation (TES) have been able to also show to influence neocortical structures, induce rhythmic resonance, phase-specific entrainment and bias the timing of neuronal spiking patterns[9–13]. These findings support the existence of cortical populations with lower action potential thresholds than those considered minimally necessary to influence ongoing local coupling and long-range oscillatory brain processes[14–16]. In the same vein, very low pulsatile magnetic fields (peak magnitudes ~0.5V/m) applied to rodent models in vivo have shown an influence on online electrophysiological evoked spiking activity[17], long-lasting hyperpolarization of the resting membrane potential of pyramidal cortical neurons[18] and signs of cortical plasticity[19]. Moreover, unique attempts to characterize the human physiological mechanisms subtending repetitive TMS at weak intensity (20, 35 or 50V/m) revealed online increases of local synchronization and corticospinal inhibition[20,21]. Similarly, weak magnetic

pulses preceding a supra- threshold TMS pulse on M1 used in short intracortical inhibitory (SICI) protocols as pre-conditioning stimuli (CS) before the test stimulus (TS) onset have shown decreases in the amplitude of motor evoked potential (MEP)[22,23]. Such short-lasting inhibitory effects may reflect the combination of axonal refractoriness[24,25], 'shunting'[26] and/or the synaptic inhibition of excitatory populations, likely mediated by a direct activation of inhibitory GABAergic interneurons characterized by lower firing thresholds[27,28]. On such a basis, it has been hypothesized, although not yet fully demonstrated -either directly or indirectly- that weak magnetic fields may selectively engage intracortical inhibitory mechanisms and suppress cortical excitability. Overall, a more detailed characterization of the evoked signatures of weak magnetic pulses at different intensity levels, and their mechanisms of action remains paramount to better understand the effects of TMS and gauge the future potential of weak intensity fields.

Reflecting transient changes in excitatory/inhibitory balance traceable with concurrent EEG recordings, the modulation of cortical oscillations with concurrent rhythmic TMS offers a unique non-invasive tool to probe neuromodulatory effects at different intensity levels[29]. Here, we investigated the *online* TMS-EEG signatures of dose-dependent (input-output) responses driven by brief frequency-specific rhythmic TMS bursts encompassing a full range of individualized subthreshold TMS intensities (from 10-100V/m) and used such evidence to speculate on the relationship between the former and the engagement of neuronal subpopulations priming excitatory or inhibitory intensity-dependent EEG signatures. On the basis of prior rodent and human literature from our team, we hypothesize that weak intensity pulses may primarily show oscillatory entrainment of natural endogenous rhythms, while high intensity stimulation close to motor threshold will impose external frequency rhythms.

MATERIALS AND METHODS

Participants

A small cohort of healthy volunteers (n=4, mean age ±SD = 26.1 ± 5.8 years; 2 females / 2 males), all right-handed according to the Edinburgh Handedness Inventory[30], free of neurological and psychiatric symptoms were recruited for this study. All participants satisfied international TMS and magnetic resonance imaging (MRI) safety criteria[31,32] and provided informed consent prior to their participation in the study. The research protocol followed the Declaration of Helsinki, and French laws for good clinical practice (ICH-E6, R2) and received the approval of a local ethical committee (*Comité de Protection des Personnes, Ile-de-France I*).

Experimental design

First, all participants carried out an MRI imaging battery session, including a scan, needed for TMS neuronavigation which took place at the ICM's CENIR MRI platform. Then participants were invited to participate in a TMS-EEG session during which TMS was applied to their dominant left primary motor cortex (M1), while EEG was concurrently recorded to characterize evoked cortical responses to 5 stimulation conditions consisting in the delivery of active (aka. real) or sham short 20 Hz TMS bursts at 10% 30%, 50%, 70% and 90% of the resting motor threshold (rMT). The order of the blocks was randomly counterbalanced across participants to limit the best we could, given the limited sample of participants, order bias effects (see Figure 1).

Figure 1. Experimental Design.

Transcranial Magnetic Stimulation (TMS)

Stimulation was applied over the cortical hotspot of the right First Dorsal Interosseous (FDI) hand muscle with a 70 mm figure-of-eight coil connected to a biphasic rTMS stimulator (Magstim Rapid 2). During the session, TMS coil position was tracked using an MRI-based frameless stereotactic neuronavigation system (Brainsight, Rogue Research) and kept within a 2mm radius from the center of the targeted site on M1. Prior to the start of the session, we

documented for each participant their rMT. To this end, single TMS pulses were delivered over the M1 hotspot of the right FDI recorded muscle monitored with EMG (Signal 5.0, Cambridge Electronic Design Limited, Cambridge, UK). Once the FDI hotspot was identified and labeled using an MRI-based frameless neuronavigation system, the precise rMT was estimated for each participant, defined as the lowest TMS intensity able to induce at least 5 out of 10 MEP (50%) of a minimal peak-to-peak amplitude of 50µV.

During the main experiment and for each of the tested TMS intensities, two rounds (from now on 'blocks') of 60 trials delivering 4-pulse-bursts at a beta frequency of 20Hz (inter-stimulus interval (ISI) of 50ms with a total burst duration of 150ms) were administered to the left M1 cortex. Each block included 40 active TMS bursts and 20 sham TMS bursts randomly embedded during the former, hence a total of 120 trials (80 active and 40 sham) per participant and TMS intensity condition. A variable inter-trial interval (ITI) between 4-7 secs was established to limit unlikely potential carry-over effects. Sham TMS bursts were delivered with a second rTMS machine (Magstim Super Rapid^2) using an identical figure-of-eight TMS coil (70 mm double coil) placed perpendicularly in contact with the dorsal aspect of the active TMS coil positioned on M1. Participants were seated in a comfortable armchair with their heads placed on a chin rest at 57 cm from a LCD monitor (15 inches) and required to fixate their gaze in a central white cross on a dark background, limiting as much as they could eye blinks during and immediately preceding or following each TMS burst.

TMS-EEG data acquisition

EEG was continuously recorded with TMS-compatible EEG amplifiers attached to two 32 real electrode grids mounted on an elastic EEG cap (ActiChamp, Brain Products) following the International 10–10 EEG system. FPz and Fz served as ground and reference respectively. Impedances were kept below 5kΩ during the experiment and signals were sampled at 25kHz. To limit TMS-related auditory evoked EEG components participants wore foam earplugs through

which white noise was continuously played (ER-3C insert earphones, ETYMOTIC Research) at an individually tailored volume ensuring the masking of TMS clicking sounds.

TMS-EEG pre-processing

Signal preprocessing was performed using in-house-custom MATLAB scripts (The MathWorks Inc., Natick, MA, USA), EEGLAB functions (UC San Diego, La Jolla California, USA; [33]) and an *ad hoc* TMS–EEG signal analyzer plugin (TESA;[34]). EEG time series were epoched around the 1st pulse of each TMS burst [-1.200ms,1.200ms]). Electromagnetic artifacts generated by individual pulses and recharging artifacts were removed and zero-padded ([-2ms,10ms]) and the missing signal was interpolated using a spline function[35]. Following, data was downsampled to 5KHz and decomposed, after excluding 'bad channels', with Independent Component Analysis (ICA) (ICA, EEGLAB 'runica' algorithm, 60 maximum components). Ocular components were identified as artifacts based on well-established guidelines[36]. These procedures led to the rejection of an average of 6±3 (mean±SD) components. Next, data was high-pass filtered with a fourth-order backward Butterworth filter (2Hz), processed with the SOUND algorithm to suppress recording noise, and with the SSP-SIR algorithm to suppress remaining TMS-evoked muscle artifacts[37,38]. Missing (i.e., zero-padded) signals were interpolated, and a second ICA was computed to remove possible residual artifacts. Datasets were bandpass filtered with a fourth-order Butterworth filter ([2-85Hz], downsampled to 1Kz, baseline corrected and re-referenced to the average. Finally, EEG time series were spatially filtered to recover signals emerging locally from directly stimulated brain regions with an E-field based spatial filtering, EPICURUS, procedure, developed by our team.

EEG Data analyses

All analyses were implemented using the Fieldtrip[39] Matlab-based toolbox. To identify signatures of TMS intensity dependent oscillatory entrainment or the modulation thereof, we

explored as metrics the modulation of Event-Related Spectral Perturbation (ERSP) also referred to as 'elicited' oscillations; and of Inter-Trial Phase Coherence (ITPC). For the analysis of ERSP, EEG epochs encompassing all the trials of a given TMS burst intensity condition were first bandpass filtered ([2-45Hz]). Then, signals were time-frequency transformed for the ([8-35Hz]) frequency range (i.e., surrounding stimulated frequency 20Hz) in steps of 0.01Hz using complex Morlet wavelets[40,41]. For the analysis of local entrainment analyses (i.e., ITPC), signals were time-frequency transformed for the ([8-35Hz]) using complex Morlet wavelets and the outcomes used to estimate phase synchronization across trials. Signals were Z-transformed using the mean and standard deviation of the entire epoch length ([-0.5s to 0.6s]) and baseline corrected ([-0.5s, -0.2s]). Cluster-based permutation statistics (5000 iterations) were used to compare the time series of elicited oscillatory and phase-coherence amplitudes of Real vs. Sham conditions respectively for each one of the 5 tested TMS intensities [42]. Monte Carlo p-values were calculated via a two-tailed test (threshold: $p < 0.05$). Statistical time domain was set to permute from TMS onset to epoch offset ([from 0ms to 600ms]), and restricting the tested frequencies to those (6-32Hz) surrounding the entrained beta frequency (20Hz) used during the experiment.

RESULTS

A total of n=4 subjects were finally included in this study. The session was well tolerated and no major or minor adverse events were claimed or reported. After EEG data cleaning and trial rejection, a fair amount of clean trials (~300 and ~100 samples for ACTIVE, aka REAL, and sham TMS respectively) could be included for the analyses of each intensity condition, however with large differences across conditions (424 for active 10%rMT and 126 for sham 10%rMT; 412 for active 30%rMT and 110 for sham 30%rMT; 410 for active 50%rMT and 115 for sham 50%rMT; 378 for active 70%rMT and 101 for sham 10%rMT; and 356 for active90%rMT and 94 for sham 90%rMT). Transcranial evoked potentials (TEPs) extracted from EEG time-series signals were detected for all intensity conditions except for the 10%rMT intensity bursts showing

features which differed with respect to those present during their associated sham controls, (see Supplementary Figure 1).

Changes in elicited local spectral perturbation

To test whether TMS at different intensities was able to modulate brain oscillations, we performed a cluster analysis and cluster based statistics comparing the time-frequency power spectra across conditions. To this end, ERSP cluster-based statistics were performed on comparing REAL vs. SHAM rhythmic 20Hz TMS bursts independently per each of the TMS conditions (intensity levels) applied. REAL vs. SHAM rhythmic 20Hz 4-pulse subthreshold intensity TMS bursts at 70% of the rMT yielded in the time and frequency domain a statistically significant cluster (~0-250ms, p=0.004) of spectral power increase around the beta band (15-25Hz), outlasting the 150ms covered by the TMS burst, that lost its frequency-specificity across time. Similarly, during the delivery REAL vs. SHAM rhythmic 20 Hz stimulation at 90% of the rMT (commonly used to engage modulatory effects in M1) yielded a significant time-frequency cluster (~0-250ms, p=0.004) of increased spectral power around the beta band (15-25Hz) which also outlasted the duration of the TMS burst and became broader-band as time evolved (see Figure 2). No significant clusters in the time and frequency domains were observed for 50, 30, nor 10%rMT intensity bursts. However, TMS frequency-specific perturbations for 30%rMT early (~0-150ms) clusters during (*online*) TMS delivery and 50%rMT during an epoch covering both *online* and also immediately *off-line* aftereffects (~0-250ms), bore a resemblance with classical TMS frequency-specific perturbations elicited at near-motor threshold levels (e.g., 90%rMT).

Figure 2. Event-Related Spectral Perturbation.

Changes in local phase synchronization

To test whether TMS 20 Hz beta bursts at different intensities were able to entrain activity synchronizing local M1 EEG generators, we performed a cluster analysis comparing inter-trial phase coherence (ITPC) during ACTIVE (aka REAL) vs SHAM conditions. To this end, as for prior analysis on ERPS, we here performed cluster-based statistics on ITPC contrasting REAL vs. SHAM rhythmic 20Hz TMS independently for each of the tested intensity conditions. REAL vs. SHAM rhythmic 20Hz TMS 4-pulse bursts yielded a significant time-frequency late cluster (~200-300ms, p=0.0002) of phase desynchronization (hence decreased ITPC) around the beta band (15-25Hz) at 30%rMT stimulation (see Figure 3). Similar analyses for these same type of TMS bursts delivered this time at 50, 70, and 90% of the rMT also showed statistically significant clusters in the time and frequency (~0-200ms, p=0.0002, ~0-300ms, p=0.0002, ~0-300ms, p=0.0002, respectively) of phase synchronization (hence increased ITPC) at the beta band (15-25Hz). In contrast, no significant time-frequency clusters were observed for 10%rMT intensity bursts. Importantly, changes of activity in all the above-mentioned significant clusters were present for a period encompassing the TMS bursts and beyond (~0-250ms) or after the TMS burst itself (~0-150ms).

Figure 3. Inter trials Phase Coherence

DISCUSSION

For the least twenty years, a myriad of experimental studies have suggested the ability of TMS delivered at so-called 'subthreshold activity' (hence below motor or phosphene threshold levels) induce online and offline modulation of cortical excitability, including oscillations [18,22,24,25,27]. Moreover, direct and alternating current stimulation (tDCS and tACS), popularly widespread non-invasive brain stimulation technologies, using sub-threshold cortical polarizing currents (0.1 to 1V/m E-field strength) unable to evoke action potentials, have shown an ability to modulate stochastic rhythms and induce phase-processing. Moreover, in spite of their poor focality, tDCS and tACS have been gaining momentum and popularity, supported by their ease of use, excellent tolerability and safety profile, low cost and high portability. Regardless, the

use and credibility as neuromodulation tools of equivalent technologies providing focal subthreshold magnetic pulsed currents, known as weak intensity TMS delivered at strengths well-below reference motor threshold levels (<80% rMT) to similarly influence brain activity remains largely overlooked by the non invasive brain stimulation domain.

In the present study, we employed concurrent TMS-EEG to investigate the *online* neural signatures of rhythmic beta 4-pulse bursts at 5 different levels of intensity (from 10 to 90% of the resting motor threshold) in the primary motor cortex and contiguously adjacent sensory regions. Even if still preliminary given our small sample size of four participants, here we report first empirical evidence that TMS at lower intensities, hence far from near-motor threshold intensities (e.g., 90%rMT) regularly used in TMS research and clinical applications, may evoke and modulate local oscillatory activity and synchrony *online*, hence while pulses are being delivered and also shortly beyond during the short period that ensues. The monitoring of scalp EEG measures such as changes (engagement, modulations or perturbation) of oscillatory activity via ERSP and ITPC recordings employed as proxies for changes in excitatory/inhibitory cortical state from the dominant left primary motor cortex was chosen for being highly accessible signals on which weak TMS currents could yield a measurable impact in humans.

In spite of the limited sample size tested, our findings strongly suggest that TMS intensities close to resting motor threshold levels might be necessary for obtaining a significant perturbation of spectral signals entraining TMS frequency specific oscillations. Indeed, classical signatures suggesting spectral perturbation (ERSP) and phase synchronization (ITPC) could be observed compared to sham patterns at 70 and 90% rMT intensity stimulation. Interestingly, TMS at 50% and 30% rMT but not levels below (10% rMT), both rarely used in the field, also showed signs of frequency-specific entrainment and phase-synchronization when active and sham TMS patterns were compared.

Prior research in non-human primates has reported that TMS pulses delivered at low intensity may evoke measurable evoked activity in intracranial recordings[43]. Moreover, recent studies in humans have contributed evidence showing that weak intensity TMS pulses at ~30V/m and ~50V/m, respectively) might be sufficient to entrain alpha cortical rhythms in the left parieto-

occipital cortex[20,21]. Our current results extend these same findings to a new cortical region - the left primary motor cortex- and to an untested TMS frequency in the beta band -20 Hz- which is 'natural' in the region, suggesting for the first time that TMS at levels below of its conventionally used intensity (around 90-100% rMT) may produce oscillatory effects of a similar nature, even if most likely in a more spatially restricted manner and not necessarily with the same amplitude and latency than those elicited at higher intensities. Also, importantly, in our study, TMS bursts delivered at 30% of the rMT yielded an episodic event of local beta desynchronization after the TMS burst period. Previous TMS-EEG research and pharmacological studies[44–46] have revealed that boosting GABAergic activity in the motor cortex evokes immediate (~0-100ms) events of alpha synchronization while desynchronizing late beta activity (~200-400ms). Similarly, neuroimaging studies in the motor control domain [47–52] have generated evidence supporting beta desynchronization during the execution of a previously planned movement, considering this spatio-temporal frequency-specific pattern a sign of increased local inhibitory activity in sensorimotor networks. Despite our ITPC analyses provide insights into oscillation frequencies that are coherent across trials, in our experiment, beta desynchronization could have a similar GABAergic origin and hence suggest that weak magnetic fields (e.g., 30% rMT stimulation) may facilitate inhibitory spontaneous activity in the primary motor cortex. Despite notorious similarity in the predominant frequency of the modulated rhythms here stated, we can not rule out the possibility that pharmacological, motor control, and TMS-EEG studies could have induced or silenced brain activity through different mechanisms of action, embroiling our ability to fairly compare these experimental scenarios. Additionally, effects on ITPC measures suggest that depending on the 'brain state', expected frequency-specific entrainment occurs at weak intensities in some scenarios, whereas in others, identical stimulation strength results in across-trial phase desynchronization hence produces no trace of entrainment effects. In other words, it is fluctuations of brain state rather than the specific level of intensity of the TMS pattern that influences the most the ability of weak intensity TMS to elicit episodic beta entrainment. In such a case, the above stated interpretation invoking GABAergic mechanisms could prove inaccurate. Recent study by our team, comparing the impact on the left M1 of TMS single TMS pulses and

20 Hz 4-pulse bursts delivered at a particularly level of low intensity (Li TMS) there referred to as '*very-weak'* intensity (Li-TMS, peak field of 5-12V/m, 6% rMT, 3% MSO, maximal stimulator output) to those delivered at conventional (aka high intensity, Hi-TMS) suprathreshold intensity (Hi-TMS, peak field 70140 V/m, 77% rMT, 60% MSO) revealed evidence of TMS-locked alpha synchronization. Despite clear methodological differences with regards to the TMS intensities employed between the latter report and the present study, our current outcomes suggest the existence of yet another biomarker providing support for post-TMS beta desynchronization in the primary motor cortex. On the basis of existing literature we speculate this outcome may align with the phenomenological foundations of increased local inhibitory GABAergic tone, suggesting that weak magnetic fields (at levels below ~half the level of the intensity employed by those conventionally used in human experimental studies and neuromodulation therapies), may predominantly facilitate the activity of inhibitory interneurons, favoring an episode of early alpha synchronization followed by late beta desynchronization.

Further experiments characterizing *online* and *offline* signatures of weak-intensity TMS in humans, involving patterns of repetitive TMS pulses in daily periodical applications will be in any case necessary to further understand the potential and limitations of very weak to weak-intensity TMS neuromodulating cortical excitability and activity according to evoked, entrained or modulated EEG outcome measures. In parallel, to better investigate and pinpoint the mechanisms of the suspected short and long-lasting inhibitory effects of weak-intensity TMS patterns, upcoming studies should include additional cortical excitability measures informing on changes in GABAergic and/or Glutamatergic neurotransmission such as intracortical inhibition (SICI) and intracortical facilitation (ICF)paired-pulse TMS protocols.

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

The datasets produced and/or analyzed during the current study are not publicly accessible due to institutional regulations. However, they may be obtained from the corresponding author of this publication following a reasoned request.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

XC-T and AV-C conceived and designed the study with conceptual input by CL, and MTC. XC-T and CL conducted the experiments and collected the data. XC-T assisted by AV-C, analyzed the data. XC-T, and AV-C prepared the first version of the manuscript. All authors approved the final version of the manuscript.

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FIGURES

Figure 1. Experimental Design. Schematic illustration of the general experimental design of the study. Briefly, the experimental session encompassed different stimulation conditions differing in intensity. Stimulation consisted in 4-pulse bursts of rhythmic (20Hz, 150ms duration) TMS stimulation delivered in blocks at 5 different intensity levels (10%, 30%, 50%, 70%, and 90%, rMT) in a pseudo-randomized order. Every stimulation condition (intensity levels) was repeated twice totaling 10 blocks per participant (2 blocks x each intensity levels, condition). Each block included 40 REAL (aka ACTIVE) 4-pulse bursts, and 20 randomly embedded SHAM 4-pulse bursts, hence a total of 80 REAL TMS bursts and 40 SHAM bursts per TMS condition and participant. rMT: resting Motor Threshold.

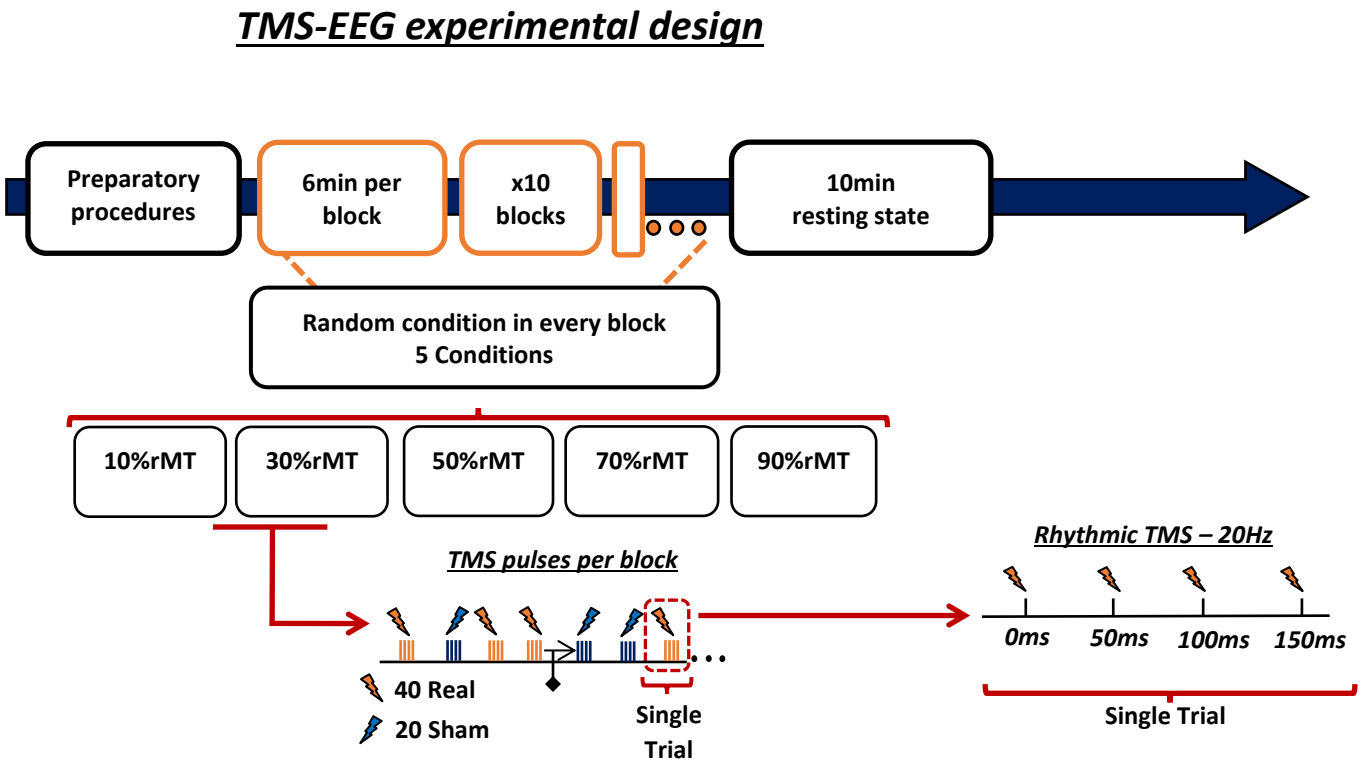


Figure 2. Local modulation of Event-related spectral perturbation (ERSP) by different TMS burst intensities. Changes in the local (stimulated M1 region) spectral power of Event-Related Spectral Perturbations (ERSP) across TMS intensity conditions. Trials have been averaged across each individual participant and trial type grouped by REAL (aka. ACTIVE) or SHAM TMS intensity conditions (10%, 30%, 50%, 70% and 90% rMT). ERSP data are displayed in the figure as Z-transformed (relative to minimum and maximum power for every condition) of ERSP group grand averages comparing REAL vs. SHAM 4-pulse equivalent TMS bursts independently for each intensity condition. Outlines (thin black line) within time-frequency ERPS figures corresponding to each TMS intensity indicate the presence of statistically significant EEG time-frequency clusters ($p<0.05$, two-sided). MT: resting Motor Threshold.

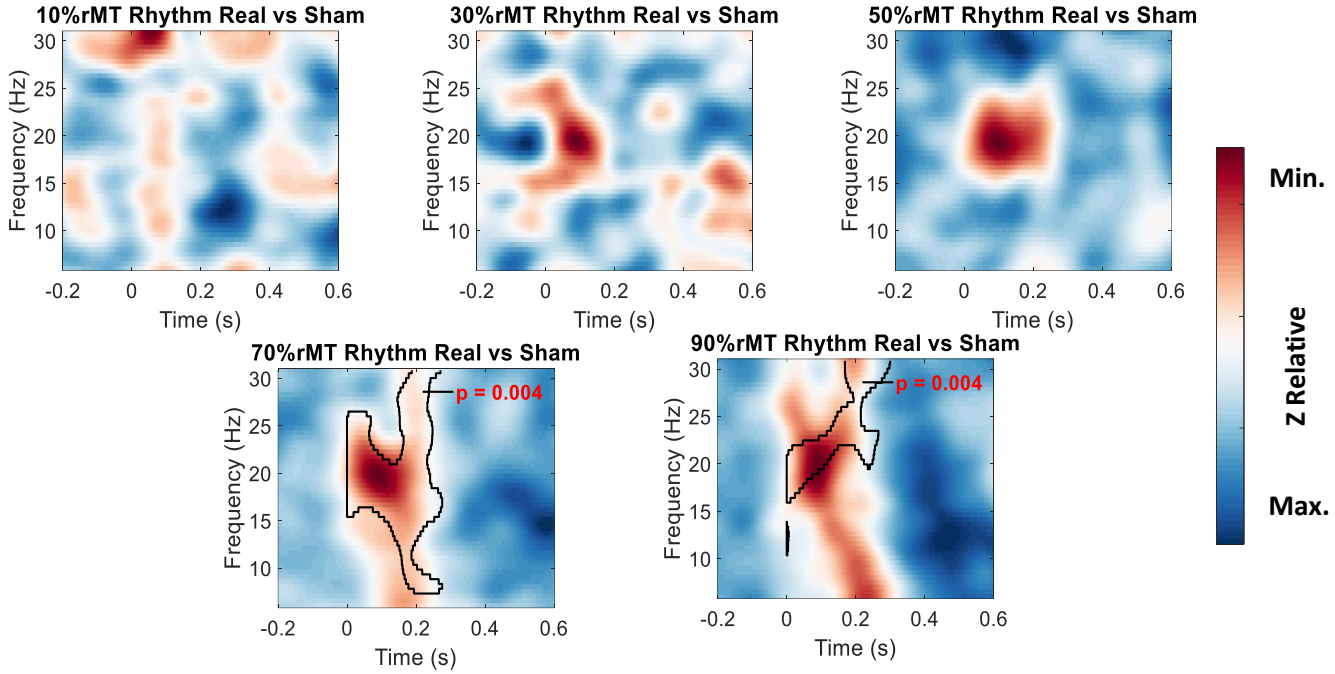
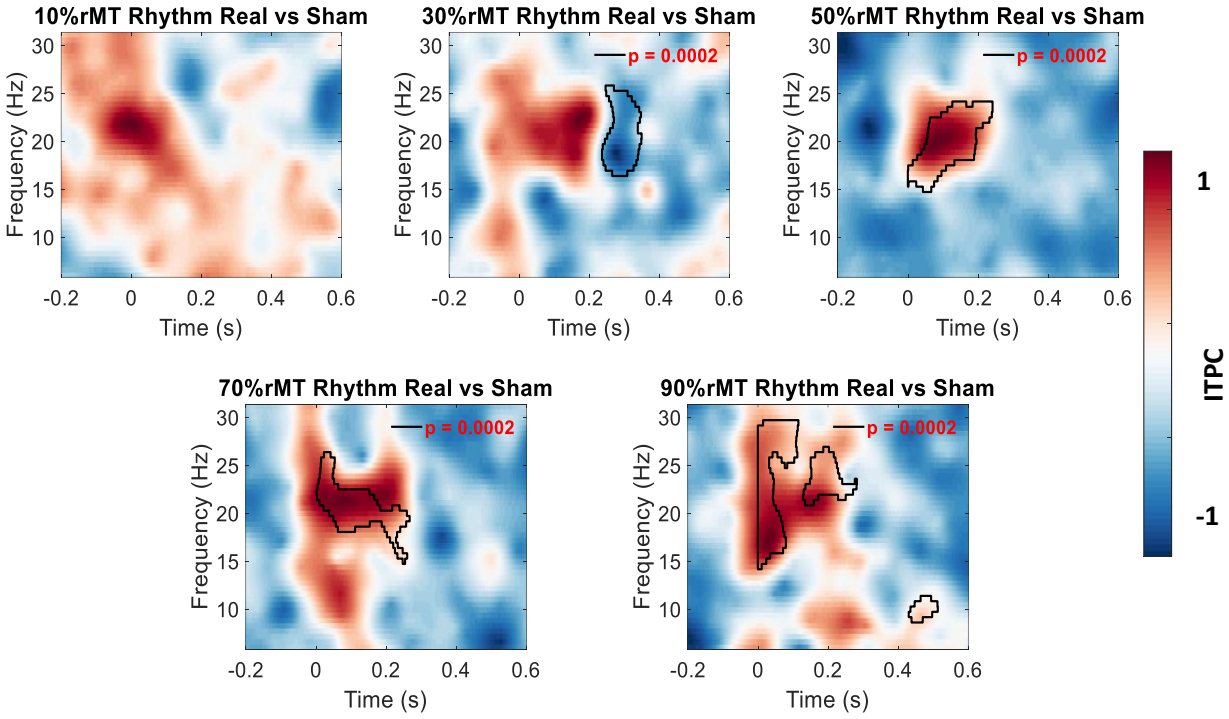


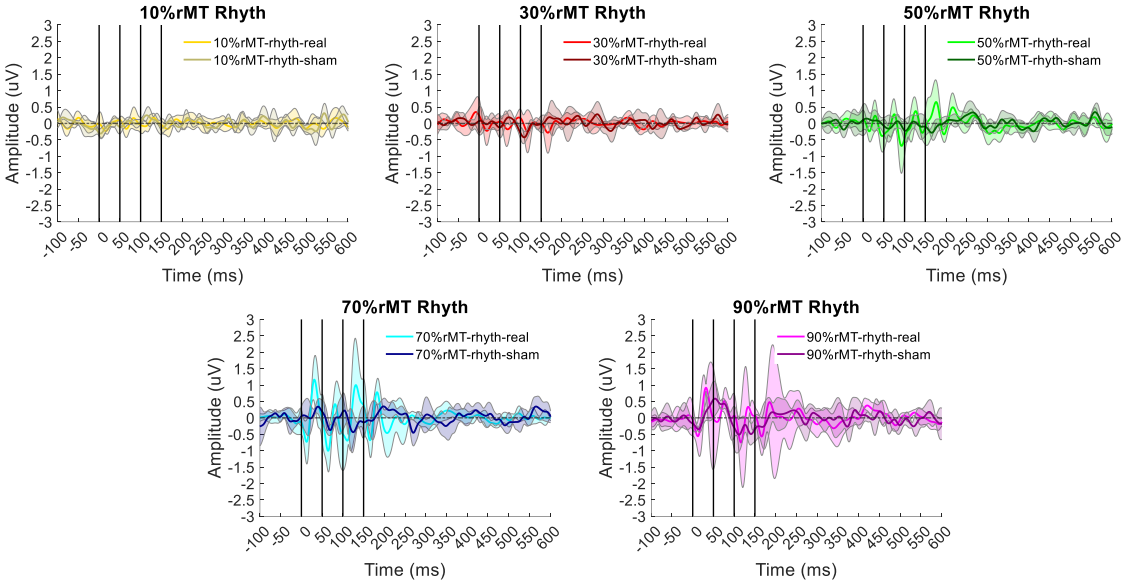
Figure 3. Local modulation of Intertrial phase coherence (ITPC) by different TMS bursts intensities. Changes in the magnitude of Intertrial Phase Coherence (ITPC) across TMS intensity conditions. All Trials have been averaged across participants (n=4) and trial type and grouped by REAL (aka. active) or SHAM TMS across each intensity condition (10%, 30%, 50%, 70% and 90% rMT). ITPC data displayed in the figure are displayed in the figure as Z-transformed ITPC group grand averages comparing REAL vs. SHAM 4-pulse equivalent TMS bursts independently for each intensity condition. Outlines (thin convoluted black line) within time-frequency ITPC figures corresponding to each TMS intensity indicate the presence of statistically significant EEG time-frequency clusters ($p<0.05$, two-sided). rMT: resting Motor Threshold.



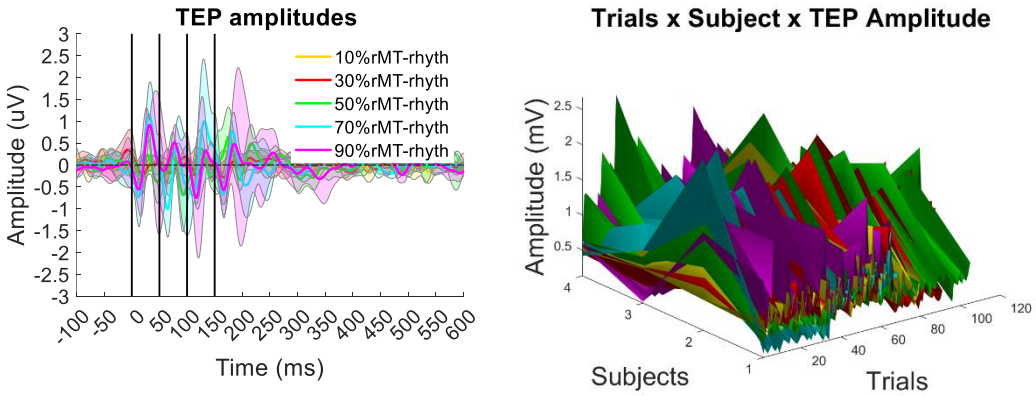
SUPPLEMENTARY FIGURES

Supplementary Figure 1 (Top Panel A) Time-courses of Transcranial Evoked potentials (TEPs) evoked by TMS bursts at different intensity conditions tested. All trials have been averaged across participants and grouped by each specific REAL (aka. active) or SHAM TMS conditions. TEPs were calculated by averaging relevant EEG epochs ([-0.5s to 0.6s]) preceding and following each TMS pulse onset (t=0) independently for each TMS intensity condition. EEG signals (in microvolts/ms) were Z-transformed using the mean and the standard deviation (SD) of the entire epoch length ([-0.5s to 0.6s]) and baseline corrected ([-0.5s, -0.2s]). The group mean (n=4) TEP time course for each TMS intensity condition is presented on each figure with a colored solid profile for REAL and SHAM trials. A thinner colored profile indicates the standard deviation (±SD) at each time/voltage value and condition respectively. Vertical lines signal on each figure the onset time of each individual TMS pulse integrating the 4-pulse TMS burst (Bottom Panel B, Left). Composite figure displaying the mean TEP time course for the group of n=4 participants tested independently for the 5 TMS intensity conditions. (Bottom Panel B, Right) Composite figure displaying the mean TEP time course independently for the 5 TMS intensity conditions and each individual participant. rMT: resting Motor Threshold.

A.Transcranial evoked potentials (TEP) across intensities



B.Overview of TEP across intensities, trials and subjects



4.2.3 Manuscript 5: May repetitive Transcranial Magnetic Stimulation at 'weak' intensity transiently modulate motor excitability? Preliminary evidence from a few cases

May repetitive Transcranial Magnetic Stimulation at 'weak' intensity transiently modulate motor excitability? Preliminary evidence from a few cases

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Dear Editor,

Developed in the late eighties, Transcranial Magnetic Stimulation (TMS) has become in hardly three decades a widely used non-invasive brain stimulation (NIBS) tool to causally explore brain function and treat neuropsychiatric conditions. Pursuing such goals, the notion that high TMS intensities (either 'near-threshold' or 'supra' threshold) on a given target area is compulsory to activate, interfere, entrain or modulate ongoing cortical activity has become highly prevalent within the TMS community. Consequently, in the primary motor cortex (M1), repetitive TMS (rTMS) is conventionally delivered at intensities tailored to the individual resting motor threshold (rMT) of each participant or patient (often 80-90% rMT), defined as the lowest intensity required to evoke Motor Evoked Potential (MEP) with a minimally established amplitude (50µV peak-to-peak amplitude) in at least half (50%) of multiple attempts in response to single TMS pulses[1].

If traditionally, near-motor-threshold TMS intensity (inducing ~100-150V/m) has been established as a criteria for effective neuromodulation, the use of transcranial Electrical Stimulation (tES) has also demonstrated an ability to lastingly modulate brain activity with much weaker electric currents (~0.4 to 0.6V/m)[2,3]. Likewise, weak TMS fields delivered transcranially at the miliTesla range(mT) to pyramidal neurons (S1 vibrissae barrel cortex) of in vivo rodent models have shown effects on spiking activity[4,5], long-lasting hyperpolarization of the resting membrane potential[6] and transient signs of cortical plasticity[7]. Last but not least, a recent study has collected evidence in humans that low intensity 10Hz rTMS (predicted field strength of ~35V/m) induced *online* local parieto-occipital alpha synchronization during TMS delivery[8]. In a similar vein, a second study reported a decrease of cortico-spinal excitability, assessed by MEP amplitude shifts, immediately following a 10 Hz rTMS[9].

Such a scarce but solid body of preliminary evidence allows now to firmly hypothesize that magnetic pulsed fields well below threshold levels may consistently modulate cortical excitability and justify new experimental endeavors to further prove it and characterize such effects. We here aimed to explore for the first time in the human primary motor cortex the effects of weak intensity rTMS at on motor cortical excitability, using similar patterns (10Hz rTMS

trains, at 10%rMT) to those shown to induce suppressive effects in the rodent barrel cortex[6] and known for enhancing corticospinal excitability when delivered at conventional intensity[1].

Four right-handed (defined by Edinburgh Handedness Inventory) healthy adult participants (n=2 Females and n=2 Males, Mean Age: 23±4 year/old) meeting safety criteria to receive TMS were included in this study. A single session of MRI neuronavigated (Brainsight, Rogue Research) stimulation was delivered over the left M1 with a rTMS device (Magstim Super Rapid2) and a standard double coil (Magstim, 70 mm figure-of-eight). Prior to rTMS, we localized the optimal cortical hotspot in the left primary motor cortex (M1) to elicit MEPs on the right First Dorsal Interosseous (FDI) hand muscle recorded with surface EMG electrodes (Signal 5.0, CED Ltd, Cambridge, UK). Following, the individual rMT was determined as the lowest TMS pulse intensity able to induce at least in 50% of the attempts (10 positive responses meeting criteria out of 20 attempts, verified twice) eliciting a peak-to-peak amplitude of at least 50µV. Three of the included participants (n=4) received 10 Hz rTMS at 10%rMT (40 trains of 40 pulses, 40 secs inter-train interval for 28 minutes). The 4th participant (n=1) was included as an active control and received the same pattern at 50%rMT.

The amplitude of Motor Evoked Potentials (MEP) in response to single TMS pulses (Sp-MEP) at intensities adjusted to evoke a ~1mV at baseline was used to monitor corticospinal excitability changes before vs after rTMS. Additionally, two paired pulse paradigms, Short Intracortical Inhibition (SICI) and Short Intracortical Facilitation (ICF) were employed to assess changes in the Excitatory/Inhibitory (Glutamatergic/ GABAergic) balance. For the SICI protocol, a subthreshold conditioning stimulus (at 65% rMT, CS) was followed after a *short* interstimulus interval (ISI, 2ms) by a suprathreshold test stimulus (TS), adjusted to evoke at baseline a ~1mV amplitude MEP. The ICF protocol followed the same approach but implementing this time, a *long* ISI (14ms).

Each of the two assessments (pre rTMS/baseline and post TMS) accrued 15 samples with a variable 4-7s inter-trial interval, hence produced a total of 15 x SICI, 15 x ICF and 15 x Sp-MEP measurements. Coil position and orientation (yaw, pitch and roll) were tracked and kept

steady throughout the session using an MRI-based frameless stereotactic neuronavigation system. During the whole session, white noise individually tailored in volume was played to participants via foam earplugs (ER-3C insert earphones, ETYMOTIC Research) to mask TMS clicking sounds. Potential changes in corticospinal (sp-MEP) and intracortical (SICI, ICF) excitability following (vs. prior) rTMS were assessed by estimating output mean MEP amplitudes and comparing them statistically with paired samples t-tests (two-tailed, $p<0.05$) with a Shapiro-Wilk to confirm normality of the data distribution.

The modulation of Sp-MEP amplitudes post vs. pre rTMS for the n=3 participants stimulated at weak TMS intensity (10% rMT) did not reach statistical significance ($U=103$, $p=0.7$) of cortico-spinal excitability (see Figure 1.A). In contrast, the amplitude of MEPs preceded by a conditioning stimulus with a long ISI indicated a significant ($t(28)=2.2$, $p=0.03$) decrease of the SICI, potentially revealing a decline of GABAergic tone. Likewise decreases in MEP amplitude revealed for ICF by comparing post vs pre 10Hz rMT rTMS records may indicate, according to some pharmacological studies, a significant ($t(28)=3.1$, $p=0.004$) decrease of intracortical glutamatergic transmission. Importantly, the analysis of the same parameters for a single active control participant (n=1) modulated this time with 50%rMT 10Hz rTMS pattern showed a statistically significant ($U=33$, $p<0.001$) increase Sp-MEP amplitudes, suggesting an enhancement of corticospinal excitability driven by a subthreshold level of rTMS stimulation rarely used for neuromodulation (see Figure 1.B). Additionally, MEP amplitudes for SICI outcomes, likely informing on GABAergic intracortical tone, were not significantly modulated by 50%rMT 10Hz rTMS ($U=98$, $p=0.55$). In contrast, MEP amplitudes associated with ICF increased significantly following rTMS ($t(28)=3.53$, $p<0.001$), an effect that could be potentially interpreted as a facilitation of intracortical Glutamatergic neurotransmission.

Previous studies employing conventional TMS intensities have reported high levels of inter-individual variability of corticospinal excitability modulations following rTMS[10], and largely attributed to head/brain anatomical variability affecting electric field distribution (E-field) and target engagement. Accordingly, differences in shape, thickness/volume of the tissue layers

TMS pulsed fields need to go through to reach the cortical gray matter may result, in case of dramatic anisotropies caused by lesions, in predicted E-fields of very different strength (V/m) and spatial distribution. We here hypothesize, that independently of the rTMS protocol applied, weak E-field strengths -as the ones delivered at 10%rMT rTMS- should affect primarily the neural populations that are the most sensitive to external perturbations (e.g., inhibitory-like interneurons).

The current proof-of-concept study, rTMS at 10%rMT suggests decreases for both SICI and ICF MEP amplitudes, indicating that weak intensity rTMS perturbs intracortical excitability modulation systems, with a combined but non-synergistic effect on GABAergic and Glutamatergic systems, whereas cortico-spinal excitability changes estimated by sp-MEP amplitude remained steady. This outcome aligns with those reported in rodents or humans with similar 10 Hz rTMS patterns at 'very-weak' and 'weak' intensity, respectively by which this pattern, otherwise well-known for its net excitatory lasting effects when delivered at near-threshold intensities (80-90%rMT) might instead decrease excitability. The preliminary analysis of a single participant explored with 50%rMT rTMS 10 Hz patterns for the same amount of time suggests such a subthreshold intensity very rarely employed for neuromodulation, significantly increased corticospinal and intracortical excitability, suggesting a different mechanism of action for stimulation patterns depending on the intensity at which they are delivered.

Unfortunately, the small sample size of our preliminary study calls for caution, and at this stage we can not yet decipher whether as we hypothesized, rTMS at weak intensity exerts a rather net unidirectional inhibitory effect on the intracortical excitability of primary motor regions by down regulating intracortical Glutamatergic neurotransmission. Further work is warranted to further pinpoint the characteristics of low-intensity TMS stimulation, by assessing with sensitive electrophysiological measures, the influence of these same TMS patterns in other cortical regions, eventually delivered in periodical sessions.

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Competing interests

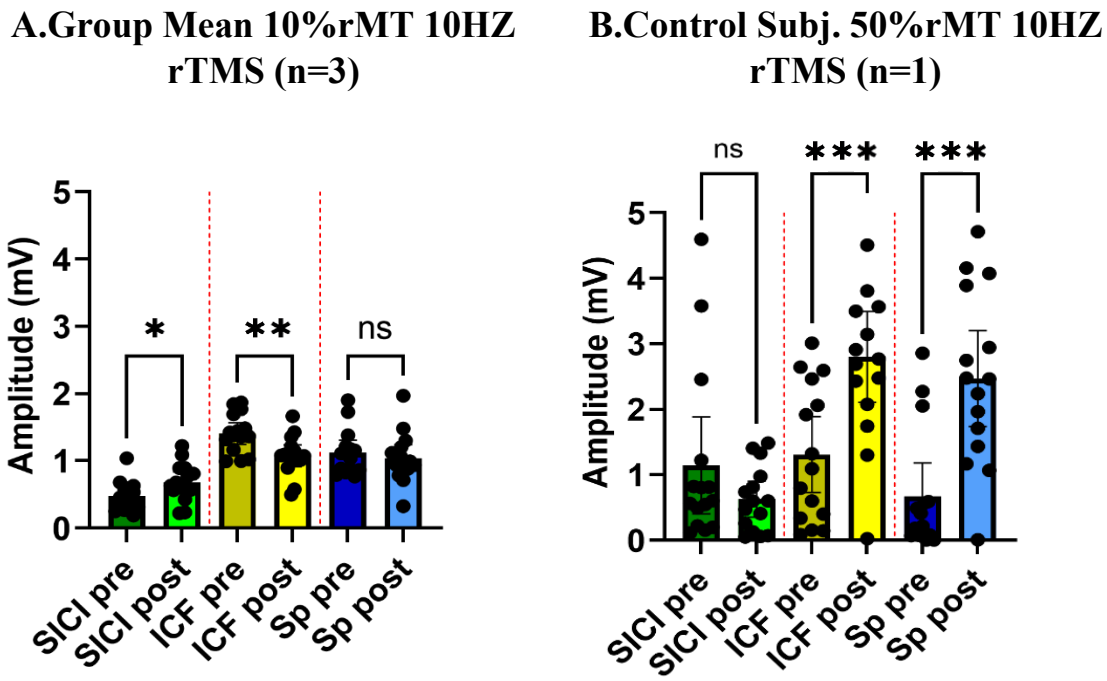
The authors declare no competing commercial or financial interests.

Acknowledgements

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FIGURE

Figure 1. Experimental results. Histograms displaying Motor Evoked Potential (MEP) amplitude (in mV) for the 3 different outcome measures employed in our study assessing cortico-spinal and intracortical excitability changes prior and following a subthreshold intensity rTMS protocol (40 trains of 40 pulses, 40 secs inter-train interval for 28 minutes) delivered to the left primary motor cortex: Single pulse MEPs (Sp-MEP), SICI (Short Intracortical Inhibition (SICI) and ICF Short Intracortical Facilitation (ICF). TMS intensity was individually tailored in our participants to 10% of their individual resting motor thresholds (10% rMT) n=3 participants) or at 50% rMT in a single case (n=1 participant) explored as an active higher intensity control. Panel A (left). Displays the MEP amplitude group average for n=3 participants stimulated with rTMS at 10% rMT. Panel B (right), displays the MEP amplitude of a single control participant (n=1) stimulated with rTMS at 50% rMT. * p<0.05, **p<0.01, ***p<0.001.



4.3. Experimental section 3 – Networked multifocal transcranial brain stimulation in post-stroke clinical disorders

Stroke lesions in the context of cerebro-vascular disease are the primary cause of long term disability in the developed world. Stroke lesions induce either ischemic or haemorrhagic damage to cortical and subcortical grey and white matter brain structures leading to significant impairments of either motor, sensory and higher level cognitive functions depending on the regions and networks affected by lesion. However, the final picture of neurological deficits suffered by a given patient and the chances for an spontaneous or rehabilitation-induced recovery process to overcome disability, does not depend on the localisation and volume of necrosis affecting neurons or passage fibers within the boundaries of the affected region. Indeed, besides local direct damage on the damaged site and the surrounding penumbra area, stroke engages large-scale so called 'diaschetic' functional activity/excitability effects in further and distant interconnected areas by virtue of abnormalities in local and distant functional interactions impairing normal communication, information transfer and processing.

Since the pioneer studies of Pierre Paul Broca on language-impaired patients in the second half of the XIX century (1861), brain damage and more specifically stroke lesions haven prove instrumental to map the functional role played by brain anatomical regions and structures, fostering progress in neurology and neurosciences. Building on ancient knowledge derived from anatomo-clinical characterization (seen introduction of this dissertation) prior to the advent of neuroimaging, integrating the game-changing role played by the development of Positron Emission Tomography (PET) and structural and functional MRI and recently integrating current uses of machine learning algorithms on large datasets, stroke cases have served to explore and map the localisation and distribution of cognitive processes and behaviours in brain systems. In a parallel vein, stroke cases have allowed the characterisation of the cellular and molecular environment at different post lesional stages facilitating or precluding adaptive plasticity and recovery. On the basis of all the accrued information, neurology is starting to learn to identify patients who might best benefit from medical and rehabilitation strategies via a reorganisation of spared cortical connections from those who might rather benefit from compensational strategies.

In this third and last Experimental section 3 of this doctoral dissertation, we explored the effects and clinical potential of noninvasive neuromodulation technologies with anodal transcranial direct current stimulation, for middle cerebral artery strokes resulting in motor impairments. Strategy-wise, rather than aiming to locally modulate the excitability of ipsilesional or contralesional (interhemispheric inhibition) structures, we aimed to innovate and assess the stimulation of target 'diaschetically' impacted networks and recover impaired motor upper limb function by facilitating in their respective cortical centres in the ipsilesional dorsolateral prefrontal cortex (DLPFC) and the contralesional anterior cerebellum (CEREB), sustained attentional and reward and planning processes and motor adaptation/learning skills.

The underlying rational is that these two structures and their associated networks oversee crucial cognitive processes facilitating motor function/recovery, while they are physically interconnected with primary motor and premotor regions as part of a larger system. Beyond our interest in clinical applications, our research contributes to the cause of characterizing sound and dysfunctional activity subtending physiological voluntary motion with regards to their interactions with prefrontal attentional/reward/

planning and cerebellar motor adaptation networks. Moreover, by placing subacute and chronic stroke patients at the core of a clinical trial design and supplementing our neuromodulation approaches with structural neuroimaging (MRI) and electroencephalography (EEG) measurements we were able to explore multidimensional correlates of compensation or recovery via plasticity mechanisms. In this context, the E-Brain protocol was designed as a pre-registered double-blind, parallel-arms, sham-controlled, randomized trial recruiting middle cerebral artery (MCA) stroke patients between 18 and 85 years old, enrolled in a 'live' rehabilitation program, who suffered a stroke 4-12 months prior to randomization. The protocol compared in separate and independently groups of patients received four anodal tDCS conditions: GROUP1: ipsilesional-DLPFC stimulation; Group 2: contralesional-CEREBellum stimulation; GROUP 3: the simultaneous combination of the two former; and GROUP 4: SHAM anodal tDCS. In all four groups, tDCS was delivered daily for 2 weeks (5 sessions per week, Monday to Friday) following a high density (HD tDCS) montage optimized on a standard brain volume via a computational solution (SIMNIBS 4.0) aiming to deliver to target at least 0.25V/m current strength the cerebellum and the DLPFC (Figure 28). Changes in motor abilities driven by tDCS stimulation were assessed with clinical scales-(National institutes of health stroke scale (NIHSS), Fulg-Meyer Assessment (FMA) focus on the upper limb which was defined as the primary outcome measure. Secondarily, impact on prefrontal and cerebellar functions were tested generally with the Montreal Cognitive assessment (MoCA) and with a specific computer-based task (visuo-motor adaptation task). All clinical tests were administered by trained clinicians at 3 time milestones: I) Baseline assessment (W0) at 2-3 days (48h-72h) before the tDCS regime onset; II) Post-tDCS assessment (W3) at 2-3 days (48h-72h) after the end of the treatment regime; and III) Follow-up assessment (W7), 30 days after the last day of the treatment. Note that the current study protocol (E-Brain), has allowed us to gather a multidimensional dataset. Nevertheless, for the purpose of this dissertation, our analysis will focus on neuroanatomical, clinical and cognitive measures relevant to estimate the impact of motor impairment and the recovery of voluntary motor function, supplemented by a preliminary analysis of EEG correlates. Details on the theoretical and conceptual framework, the methods and the registered analytical strategy of the E-Brain trial can be found in **Manuscript 6**.

Manuscript 6: *Corominas-Teruel, X., Bracco, M., Fibla, M., Segundo, R. M. S., Villalobos-Llaó, M., Gallea, C., Beranger, B., Toba, M., Valero-Cabré, A., & Colomina, M. T. (2023). High-density transcranial direct current stimulation to improve upper limb motor function following stroke: study protocol for a double-blind randomized clinical trial targeting prefrontal and/or cerebellar cognitive contributions to voluntary motion. Trials, 24(1), 783. https://doi.org/10.1186/s13063-023-07680-8.*

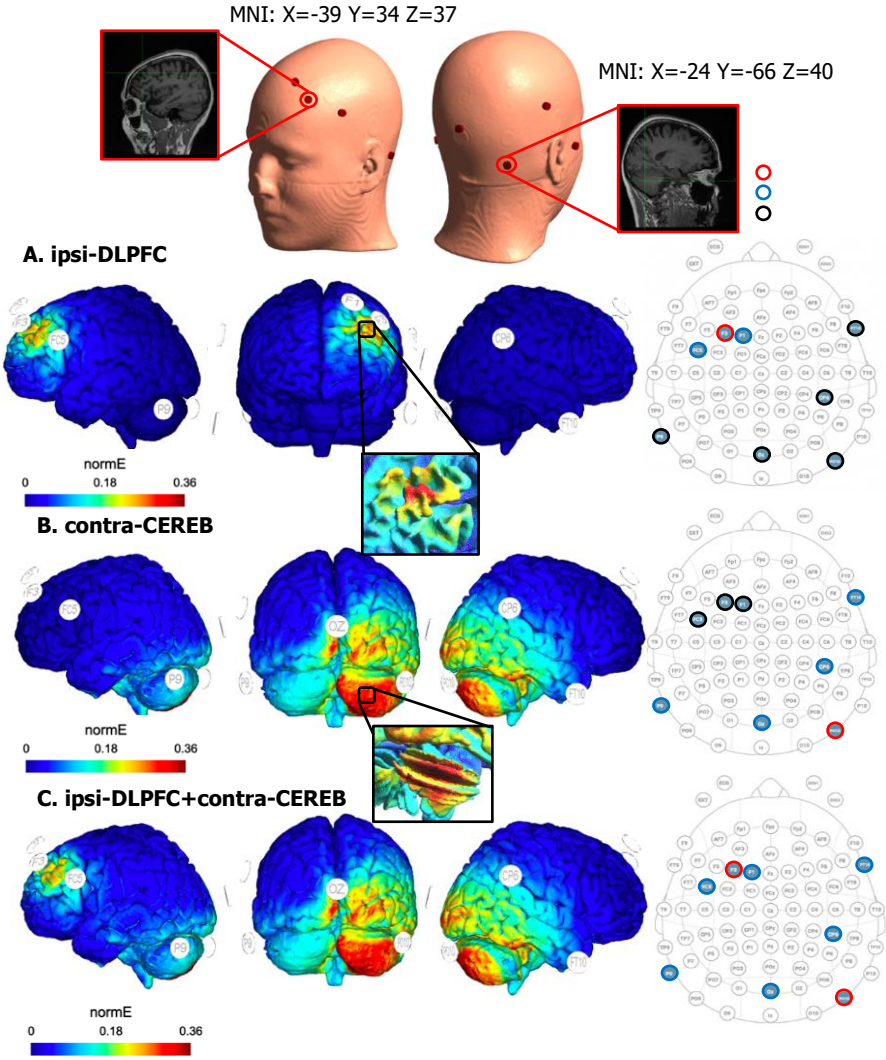


Figure 28. Illustration of the main scientific contribution in Manuscript 6. Illustration of computationally optimized tDCS montage solutions for the 3 active allocation groups of the E-brain study protocol. Manuscript 6 details the experimental objectives, protocol, and analyses of the E-brain study.

The specific objectives of the study were (1) to gauge the clinical potential of dual site vs, single site DLPFC or Cerebellar anodal tDCS to drive clinical improvement in upper limb motor function; (2) to evaluate the influence of these same active and sham anodal tDCS interventions on specific cognitive contributions to motor function associated to the stimulated targets (notably the ipsilesional DLPFC and the contrtalateral Cerebellum), and (3) identify demographic (gender, age and level of instruction), clinical (post stroke lapse, stroke type and severity of motor and cognitive deficits at baseline), lesional (gray matter damage via MRI) and electrophysiological (via

EEG) biomarkers associated to the severity of motor impairments and their modulation following the different anodal tDCS regimes tested in the trial. At the time of completing this dissertation, the study has recruited and completed stimulation and follow twenty-seven stroke patients (n=27, hence 6-7 per each of the 4 groups). They all suffered a 1st middle cerebral artery stroke resulting in enduring motor deficits and were at the chronic phase. Details on the theoretical and conceptual framework, the methods, analytical strategy and most important, the outcomes of the E-Brain trial at the current stage can be found in **Manuscript 7**.

Manuscript 7:*Corominas-Teruel, X., et al., (2024). Transcranial direct current stimulation on pre-frontal and/or cerebellar systems to improve motor function in stroke patients: Preliminary analyses from an ongoing pilot study*

In order to best characterize our stroke cohort, in patients with an available MRI (n=23 out of n=27) we outlined manually the areas of damage on each MRI section and on such basis we built in the native space a lesion mask. We then explored statistically correlations between behavioural changes and clinical improvements and lesion parameters employing low-dimensional statistical procedures. We first aimed to better understand for the whole cohort of recruited patients, hence regardless of their stimulation group, the impact of lesion features on motor severity and cognitive performance at baseline. In a second step, we employed within-group and between-groups statistical approaches to better pinpoint the clinical potential of a multitarget stimulation improving clinical functional motor and cognitive capacities and their potential relation to lesion severity. Interventions were monitored with daily safety and tolerance questionnaires. All participants tolerated the interventions and no adverse or serious discomfort events were reported. Likewise, there was not any case of drop-out, highlighting the safeties of bifocal up to 4mA stimulation. Within-group comparisons for the ipsi-DLPFC+contra-CEREB group on the MoCA general score test, showed post-tDCS scores at W7 which were marginally significantly higher than those at baseline W0 levels. Additionally, between group comparisons also revealed a marginally significant increase for post W3 vs. baseline in the upper limb FM for the ipsi-DLPFC+contra-CEREB group. These outcomes are very preliminary and isolated, hence given the low number of participants per group accrued in the E-Brain trial, they need to be interpreted cautiously and supplemented by additional data. Nonetheless, at this stage our trial validates the good tolerability of our intervention which we might contribute to support a causal implication of cerebellar and prefrontal modulations driving improvement of motor function following stroke.

Beyond purely clinical scales such as the Fugl-Meyer and the MOCA, we also considered and evaluated the impact of the stroke lesion at baseline and the modulatory effect of the active anodal tDCS conditions on more sensitive measures of cerebellar activity by means of a visuo-motor adaptation task. Baseline impairment of motor function and cognitive state did not statistically differ between groups; however, note that there was substantial variability across included participants given that minimum and maximal functional capacities were not defined as exclusion criteria. The visuomotor task revealed significant decreases in the movement deviation trajectory (hence improvements of visuo-motor adaptation performance) comparing baseline at W0 vs. post-intervention milestone W3, in all included groups with GROUP 2 delivering contralesional-cerebellar stimulation achieving the highest level. However, the combined

fronto-cerebellar stimulation group showed an increase in error variability, suggesting that the combined stimulation perturbed visuo-motor adaptation skills (Figure 29). Even if a precise circuit and mechanism subtending effective cerebellar-thalamic-frontal communication during adaptation could not yet be established, this result highlights an effect of anodal tDCS in the spared cerebellar function of these stroke patients which could potentially to motor recovery. Future research will have to add new patients to the recruited cohort and if the latter outcome is confirmed, pinpoint on the basis of MRI neuroimaging and EEG data the neuroanatomical basis and temporal dynamics of such interactions.

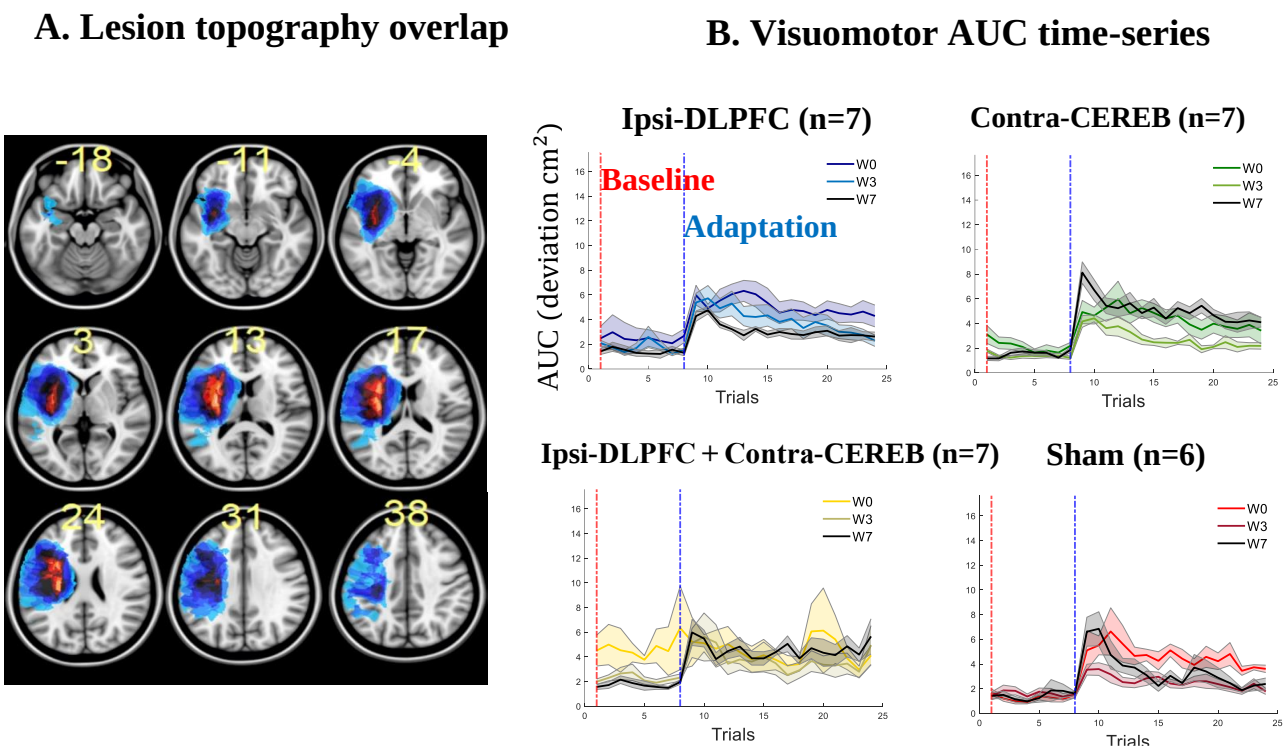


Figure 29. Summary of the main contributions included in Manuscript 7. This ongoing work addresses the isolated vs. joint contribution of prefrontal and cerebellar regions to improvement of upper limb motor function via causal modulation of such systems with anodal tDCS. **A.** Group-level lesion topography overlapping of n=21 out of 27 stroke patients recruited for the E-brain protocol. Note that stroke lesions were manually segmented and transformed to normalized space. **B.** Visuomotor Adaptation performance curves across training trials (from red to blue vertical lines) and adaptation trials (from blue vertical lines onwards) at each evaluation time-point (W0, W3 and W7) and the 4 anodal tDCS groups: GROUP1: Ipsilesional-DLPFC stimulation (top left figure); Group 2: Contralesional-CEREBellum stimulation (top right figure); GROUP 3: simultaneous combination of the former two (bottom left figure); and GROUP 4: Sham anodal tDCS (bottom right figure).

4.3.1 Manuscript 6: High-density transcranial direct current stimulation to improve upper limb motor function following stroke: study protocol for a double-blind randomized clinical trial targeting prefrontal and/or cerebellar cognitive contributions to voluntary motion. Trials

Corominas-Teruel *et al. Trials* (2023) 24:783
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Trials

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High-density transcranial direct current stimulation to improve upper limb motor function following stroke: study protocol for a double-blind randomized clinical trial targeting prefrontal and/or cerebellar cognitive contributions to voluntary motion

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Abstract

Background Focal brain lesions following a stroke of the middle cerebral artery induce large-scale network disarray with a potential to impact multiple cognitive and behavioral domains. Over the last 20 years, non-invasive brain neuromodulation via electrical (tCS) stimulation has shown promise to modulate motor deficits and contribute to recovery. However, weak, inconsistent, or at times heterogeneous outcomes using these techniques have also highlighted the need for novel strategies and the assessment of their efficacy in ad hoc controlled clinical trials.

Methods We here present a double-blind, sham-controlled, single-center, randomized pilot clinical trial involving participants having suffered a unilateral middle cerebral artery (MCA) stroke resulting in motor paralysis of the contralateral upper limb. Patients will undergo a 10-day regime (5 days a week for 2 consecutive weeks) of a newly designed high-definition transcranial direct current stimulation (HD-tDCS) protocol. Clinical evaluations (e.g., Fugl Meyer, NIHSS), computer-based cognitive assessments (visuo-motor adaptation and AX-CPT attention tasks), and electroencephalography (resting-state and task-evoked EEG) will be carried out at 3 time points: (I) Baseline, (II) Post-tDCS, and (III) Follow-up. The study consists of a four-arm trial comparing the impact on motor recovery of three active anodal tDCS conditions: ipsilesional DLPFC tDCS, contralesional cerebellar tDCS or combined DLPFC + contralesional cerebellar tDCS, and a sham tDCS intervention. The Fugl-Meyer Assessment for the upper extremity (FMA-UE) is selected as the primary outcome measure to quantify motor recovery. In every stimulation session, participants

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will receive 20 min of high-density tDCS stimulation (HD-tDCS) (up to 0.63 mA/cm^2) with π cm^2 electrodes. Electrode scalp positioning relative to the cortical surface (anodes and cathodes) and intensities are based on a biophysical optimization model of current distribution ensuring a 0.25 V/m impact at each of the chosen targets.

Discussion Our trial will gauge the therapeutic potential of accumulative sessions of HD-tDCS to improve upper limb motor and cognitive dysfunctions presented by middle cerebral artery stroke patients. In parallel, we aim at characterizing changes in electroencephalographic (EEG) activity as biomarkers of clinical effects and at identifying potential interactions between tDCS impact and motor performance outcomes. Our work will enrich our mechanistic understanding on prefrontal and cerebellar contributions to motor function and its rehabilitation following brain damage.

Trial registration ClinicalTrials.gov NCT05329818. April 15, 2022.

Keywords Stroke, Transcranial direct current stimulation, Plasticity, Neurorehabilitation, Randomized controlled trial

Introduction

Middle cerebral artery (MCA) strokes are known to cause direct structural damage to key sensory-motor networks in charge of executing and controlling voluntary motion actions in frontal and anterior parietal cortical or in associated subcortical structures. Despite the influence of lesion location and extent [1–3], the magnitude of motor performance deficits following stroke [4–6] cannot be solely explained by ischemic damage on motor systems but also by diaschetic effects altering network interactions with local and distant structures contributing substantially to optimal motor activity.

Transcranial direct current stimulation (tDCS) is among the most popular non-invasive brain stimulation approaches currently used in clinical settings. It is characterized by its portability, low cost, ease of use, a safe profile of side effects, and high flexibility to target several locations simultaneously. Transcranial DCS is based on the application of a low-intensity continuous current inducing polarity-dependent sub-threshold shifts of neuronal resting membrane potential towards (anodal) or away (cathodal) from the firing threshold [7–10].

By virtue of such effects, whereas single sessions of tDCS have shown the ability to transiently modulate corticospinal excitability, periodical sessions of such simulation promote long-term potentiation/depression-like plasticity [11]. Transcranial DCS has been used as a therapeutic intervention to boost cognitive and motor recovery following stroke in diverse settings and evaluated with regards to its ability to improve voluntary upper limb function [12–16]. Despite its success in small samples of selected participants, the effects reported by accumulative tDCS interventions in stroke have often been shown to be inconsistent when applied to larger cohorts of patients [17]. Additionally, tDCS outcomes have been found to be highly influenced by variables such as post-stroke-to-treatment-onset time-lag, lesion site and volume, a large variety of stimulation parameters (electrode location, current intensity, density, regime periodicity, etc.), and interindividual head and brain anatomical differences affecting the distribution of tDCS-generated electric fields [18, 19].

Over the last decade, classical non-invasive brain stimulation (NIBS) approaches with transcranial magnetic stimulation (TMS) or with tDCS have privileged clinical strategies based on either the upregulation of directly affected primary motor systems or the downregulation of homologue motor networks of the spared contralesional hemisphere linked with the former via inhibitory trans-callosal inter-hemispheric projections.

Nevertheless, the neural signature of MCA lesions has revealed strong interactions with alterations in sustained attention (engaging prefrontal and fronto-parietal systems) and large-scale desynchronization phenomena, both key factors limiting motor function or precluding recovery after brain damage [20–22]. More specifically, the severity of motor deficits has been associated with the strengthening of impaired inter-hemispheric functional connectivity between the dorsal attention network (DAN) and sensory-motor networks (SMN) [23–26] and high- and low-frequency oscillatory abnormalities (decreases and increases respectively) in the injured hemisphere [27, 28]. Likewise, a connectome-based predictive model exploring fractional anisotropy (FA) in stroke has highlighted the role of the ipsilesional dorsolateral prefrontal cortex (DLPFC) and cerebellar areas subtending motor symptoms and suggested their potential to convey motor recovery when manipulated with NIBS interventions [29]. In such a context conventional therapeutic neurostimulation directly modulating the excitability of damaged areas in charge of mapping lost functions has shown inconsistent outcomes. Heneforth, recent innovative approaches [30, 31] have been focused on

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reversing abnormal large-scale network signatures by inducing synergistic effects from spared cortical regions contributing to impaired function [30, 31].

On such basis and superseding the clinical limitations of traditional approaches [32–34], we here aim at providing evidence that a multi-site tDCS stimulation approach targeting simultaneously the dorsolateral prefrontal cortex and the anterior lobe of the cerebellum will be able to drive significant clinical improvement in upper limb motor function compared to a placebo intervention, and also that such effects would be greater than the isolated stimulation of either site individually. Importantly, a battery of secondary measures relying on cognitive tasks assessing cerebellar and prefrontal contributions to motor function, sustained and selective attentional processes, EEG recordings and structural MRI neuroimaging will inform on some of the neural mechanisms involved in such recovery.

Study objectives

The main and primary "key" objective pursued in this clinical trial is to (1) identify the clinical potential of the multitarget cortical stimulation by comparing the effect of three tDCS interventions (anterior cerebellar lobe stimulation, dorsolateral prefrontal stimulation and the combination thereof) driving clinical improvements of upper limb motor function in stroke patients, as assessed by the Fugl-Meyer Assessment (FMA). We specifically hypothesize (key hypothesis of the study) that combined stimulation of the former two cerebral sites will enhance clinical recovery compared to sham stimulation and also to the stimulation of either site (anterior cerebellar or dorsolateral prefrontal) in isolation.

The secondary objectives of our clinical trial are: (2) to evaluate the influence of such interventions on specific cognitive and motor processes sensitive to the contribution of the modulated targets, i.e., via an impact on sustained attention and cognitive control (dorsolateral prefrontal tDCS) or motor adaptation skills (anterior cerebellar tDCS); (3) to explore whether clinical improvements are induced by isolated interventions or if their combination can be associated to the normalization of neurophysiological outcome measures (increased local and/or inter-areal synchronization or changes of abnormal neural states, taken as proxies of enduring adaptive plasticity); finally, (4) to identify biomarkers (clinical and cognitive scores, neuroimaging features, and electrophysiological measures and tDCS current distribution modelled parameters) associated to the severity of motor impairments and their improvement following stimulation.

Material and methods

Study settings

This trial will be conducted in the *Hospital Universitari Joan XXIII*, Tarragona, Spain. All interventions and assessments will be carried out in the Rehabilitation and Physical medicine department of this hospital. MRI acquisitions will be performed in the Radiology and Nuclear medicine department in this same clinical institution. An IRB protocol in accordance with the Declaration of Helsinki covering all its procedures and interventions with the registration number: 077/2021 (version: V.1_06/05/) has been officially approved on June 27th 2021 by the local ethics committee of the *Institut d'investigació sanitària Pere Virgili* (IISPV, Tarragona, Spain).

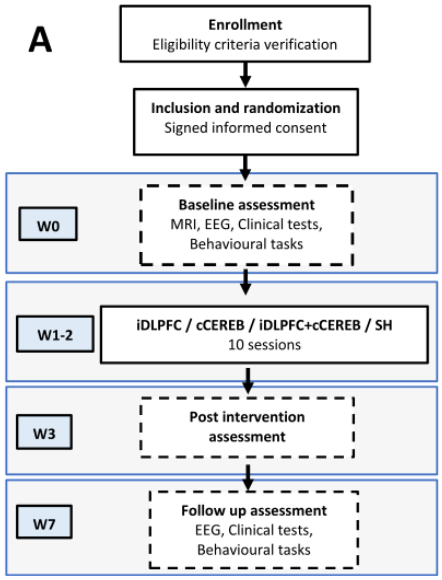
Study design

The present clinical protocol (hereafter entitled *E-Brain*) is designed as a double-blind, parallel, sham-controlled, randomized clinical trial.

Study summary

Participants will be randomly assigned to one out of the following 4 groups, GROUP 1: (ipsi-DLPFC) anodal tDCS stimulation of the ipsilesional dorsolateral prefrontal cortex; GROUP 2: (contra-CEREB): anodal stimulation of the contralesional cerebellar cortex (anterior lobe); GROUP 3: (ipsi-DLPFC + contra-CEREB) combined anodal stimulation of the ipsilesional DLPFC and the anodal contralesional cerebellar cortex; and GROUP 4: (SHAM tDCS) consisting in sham/placebo stimulation (see Fig. 1A).

For all groups, a daily tDCS session will be administrated for 10 consecutive days, with a regime of 5 sessions per week for 2 weeks (Monday to Friday). A conventional clinical magnetic resonance imaging (MRI, 3D-T1) recorded between the 1st and 3rd month after the stroke event will be retrieved from the participant's medical history or recorded at the the Hospital Universitari Joan XXIII, Tarragona (Spain) within 2 weeks prior to the trial onset. Clinical scales or scores, behavioral computer-based tasks, and electroencephalography (EEG) evaluations will be carried out at 3-time points or milestones: (I) Baseline assessment: 72–96 h (2–3 days) before the onset of the tDCS regime; (II) Post-tDCS assessment: 72–96 h (2–3 days) after the end of the tDCS regime; and (III) Follow-up assessment: 30 days after the end of the tDCS regime. Across sessions, all evaluations will be carried out under identical conditions (see Fig. 1A, B). The current protocol E-Brain follows the SPIRIT recommendations (see Additional file 1).



B

	STUDY PERIOD						
	Enrollment	Allocation	Post-allocation				Close-out
TIMEPOINT**	$-t_1$	0	W0	W1	W2	W3	W7
ENROLLMENT							
Eligibility screen	X						
Informed consent	X						
Allocation		X					
INTERVENTIONS							
HD-tDCS sessions				X	X		
ASSESSMENTS							
MRI		X					
EEG recordings			X			X	X
Clinical scores			X			X	X
Computer tasks			X			X	X

Fig. 1 **A** Flow diagram of the study design. The diagram presents a weekly calendar where W0 denotes the baseline assessment and the interventional starting point for a representative subject. contra-CEREB: anodal contralesional anterior cerebellar lobe stimulation, ipsi-DLPFC + contra-CEREB: anodal ipsilesional DLPFC tDCS combined with anodal contralesional anterior cerebellar lobe simultaneous tDCS, ipsi-DLPFC: anodal ipsilesional dorsolateral prefrontal tDCS, SHAM: sham tDCS stimulation, W0: week 0 (baseline assessment), W1-2: weeks 1 and 2 (interventional weeks were the 10 days tDCS treatment is executed), W3: week 3 (post-intervention assessment), W7: week 7 (follow-up assessment). **B** Standard protocol items: recommendations for interventional trials (Supplementary file 1). Schematic summary and milestones for enrolment, tDCS treatment, and assessments across the study time-line

Participant recruitment

Participants will be enrolled through the Rehabilitation and Physical Medicine department of the *Hospital Universitari Joan XXIII* in Tarragona, Spain. Only those patients attending the rehabilitation service enrolled in an active rehabilitation program (hereafter referred to as a "live" rehabilitation program) will be assessed for eligibility. An experienced licensed medical doctor (RMS) working for the protocol will initially screen potential participants fulfilling eligibility criteria. Following verification of inclusion criteria, participants willing to participate will be presented with the details of the protocol and asked to sign a consent form to be officially included in the study.

Inclusion criteria

The following inclusion criteria have been established as to be fulfilled by potential participants: (1) to have received a diagnosis of supratentorial ischemic or hemorrhagic unilateral stroke supplied by the middle cerebral artery (i.e., encompassing frontal–temporal-parietal regions); (2) to be enrolled in a "live" rehabilitation program in the Rehabilitation and Physical Medicine department of our institution; (3) to be between 18 and 85 years old; (4) to have suffered a stroke within 4 and 12 months prior to enrollment; (5) to have signed the informed consent form.

Non-inclusion criteria

Stroke participants presenting at least one of the following criteria will not be able to participate in our study: (1) unstable medical condition (e.g., affected by infections, with assisted ventilation, having suffered or actively suffering epilepsy or recurrent seizures, untreated psychiatric disorders, or being under an active treatment with sedative drugs); (2) participants presenting contraindications to tDCS according to the most current international NIBS safety guidelines [10]; (3) participants presenting cognitive impairments-such as severe aphasia or neuropsychiatric deficits-limiting their comprehension and their ability to follow instructions. The verification of non-inclusion criteria will be documented by means of an in-house screening questionnaire.

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Exit criteria

Patients will be dropped out from the study if: (1) they manifest, at any time and without the need to provide any explanation, their willingness to stop their participation in the clinical trial; (2) they are not compliant with the procedures of the study; (2) they experience severe discomfort or annoyance during their participation (i.e., insomnia, headache, etc.); or (3) in case of unexpected events that incapacitate patients to continue in the study. Data collected until a participant is officially considered a 'drop-out' will be included in analyses, hence not withdrawn from the study.

Sociodemographic data

During the baseline assessment, patients will be asked to complete to the best of their knowledge and ability a questionnaire including the following information: (1) age and sex; (2) stroke features (hemisphere affected, localization, stroke type, time since stroke event, premorbid conditions); (3) socio-educational information (marital status, academic level, occupation, leisure hobbies, technology usage, sport practice, smoking, alcohol or drug usage and ongoing medication); (4) past and ongoing stroke rehabilitation program (post-stroke-onset time, types of ongoing and completed programs, frequency, intensity, and periodicity). Stroke lesion features, details of the clinical history, and any missing medical information required to complete the above-mentioned questionnaire will be verified and/or completed by a legally authorized member of the medical team collaborating with the protocol (co-author RMS).

Additionally, the Edinburgh manual dexterity scale and the Beck's inventory will be administered to characterize the patient's laterality and assess the participant's mood, respectively.

Sample size

The current study is designed as a pilot clinical trial aiming to assess the effects of an isolated monofocal (single cortical site stimulation, i.e., either the dorsolateral prefrontal cortex or the anterior cerebellar lobe) or combined multifocal (simultaneous stimulation of the two former sites) multiday tDCS intervention using a high-density array of electrodes. Given the exploratory character of our study, no power analysis to estimate sample size was required by the local IRB committee. Instead, following standardized guidelines for experimental clinical trials, we considered the number of participants included in past similar exploratory tDCS studies warranting sufficient statistical power [35] and ensuring recruitment feasibility, and we concluded the need to include at least $n=15$ patients in each of our 4 tDCS treatment groups.

Randomization and blinding

The study will include $n=60$ patients with chronic MCA stroke, randomly assigned to one of the 4 experimental groups indicated above (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC + contra-CEREB, or SHAM tDCS). A patient randomization algorithm (*MinimPy* software running in *Python environment*, https://sourceforge.net/projects/minimpy/) counterbalancing groups by sex (Woman/Male), age (−65 years/+65 years) and stroke type (ischemic/hemorrhagic) will ensure equivalence for these three variables across the 4 experimental groups. The biased coin method is an algorithm implementing a biased coin minimization algorithm (base probability: 1, allocation ratio 1:1:1:1) [36] for sequential dynamic allocation, in which each new allocation is influenced by the current state of balance across delivered treatments [37].

An independent co-investigator not involved in the recruitment/enrollment of patients, tDCS application or in clinical evaluation will be in charge of patient randomization and condition/group allocation. The groups of patients associated with the four randomized tDCS conditions will be assigned a letter-coded name (Groups A, B, C, or D) providing no clue with regard to the ultimately delivered tDCS strategy. This same code will be used at all times for patient allocation and only an independent co-investigator in charge of randomization/allocation activities will be able to associate each of the codes (A, B, C, and D) to a specific tDCS condition (aka treatment group). Once a patient will be randomly assigned to a condition/treatment group, its code (A, B, C, or D) will be communicated to a team member in charge of stimulation which will simply deliver it blindly in a non-identifiable manner. To search for the potential influence of placebo or nocebo effects, investigators will debrief at the end of the follow-up, with patients and ask them to guess which stimulation group they believed they had been allocated to (allocation perception).

Double-blind (both the participant and the investigators in charge of stimulation or evaluation tasks will be unaware of the stimulation condition) will be ensured by a *blinding option* available on tDCS equipment and associated control software (*Starstim-8*® and *Neuroelectrics Instrument Controller*®, Neuroelectrics, Barcelona, Spain). An investigator (co-author MF, referred hereafter as the 'administrator') will program and blind the different HD-tDCS protocols in our *Neuroelectrics Instrument Controller* system, whereas a second researcher (co-authors XC-T, MV-L MTC, or AV-C, referred to as the 'operator') will perform the intervention sessions without knowledge of the tDCS protocol being delivered. Likewise, the operator will be in charge of EEG and (both the participant and the investigators in charge of stimulation or evaluation will be unaware of the stimulation

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condition) assessment performance. Finally, data analysis will be performed by a fourth co-investigator who will remain blind to the specific tDCS strategies implemented in each treatment condition (A, B, C, or D) until all analysis work is completed.

Interventions

tDCS electrode montage

Prior to the design of the present clinical Trial, we optimized a HD-tDCS electrode montage solution to be able to target simultaneously the DLPFC and the anterior lobe of the cerebellum using an 8-channel tDCS (Starstim, NE) equipment. The optimized computational model was generated in MATLAB (R2019a, Mathworks, USA) and SimNIBS 3.2.3 [38], an open-source package for the simulation of non-invasive brain stimulation electrical field based on participant's MRI volumes using a standard head/brain volume MNI152 (version 2009a) as template (available through the open dataset of SimNIBS). The "lead field matrix" computation defining the scalp localization of tDCS electrodes was based on the 10/20 EEG system and πcm^2 predefined tDCS electrode size. The MNI152 standard head model was reconstructed with the *headreco* routine relying on SPM12 and CAT12 for segmentation. Isotropic conductivity values were set as follows (in S/m) based on previous studies [39, 40]: WM 0.126, GM 0.275, CSF 1.654, bone 0.010, scalp 0.465, eye balls 0.500, compact bone 0.008, spongy bone 0.025, blood 0.600, muscle 0.160. The selected electrode solution (in terms of electric field focality, intensity, and electrode compatibility with an 8-channel tDCS *NE Starstim* device) was obtained by optimizing the electrode positions of each cerebral target separately (ipsi-DLPFC and contra-CEREB) and merging these solutions in a simulation of combined stimulation scenario (ipsi-DLPFC + contra-CEREB).

For an optimal simulation of the left (ipsilateral) DLPFC (MNI $x = -39$ $y = 34$ $z = 37$ to influence the Dorsal Attentional Network, DAN), we obtained the best solution limiting the total number of electrodes to 3. Additionally, given technical limitations of our ISO and CE certified tDCS device and to warrant patient safety, a total maximal current of 4 mA and a maximal individual electrode current of 2 mA were used. Ipsilesional prefrontal target coordinates (ipsi-DLPFC) were defined on the basis of previous studies ensuring a high E-field impact in Brodmann area 46 (BA46) [41]. For the computation of the optimal right contralateral cerebellum stimulation site (MNI $x = 24$ $y = -66$ $z = -40$) and to optimally impact its anterior lobe (contra-CEREB), the best solution was found with a total number of 5 scalp electrodes, a maximum current of 4 mA, and maximum individual electrode current of 2 mA[42]. We also modified the E-field *direction* controlling field strength at the target instead of a tangential orientation. Importantly, to favor spatial selectivity and avoid electrode dispersion, the contralesional temporal cortex (MNI $x = 53$ $y = -6$ $z = -41$, hence contralateral to the stimulated DLPFC) and the ipsilesional anterior cerebellar lobe (MNI $X = -9$ $Y = -88$ $Z = -44$, contralateral to the stimulated CEREB) were defined as "avoidance" regions which tDCS montages excluded from being impacted.

Current evidence from in vitro and in vivo studies has demonstrated that tDCS field strength close to 0.25 V/m are sufficient to alter neuronal excitability via modulation of its resting-state potential [9, 43, 44]. Due to the dual-site experimental approach tested in our study (simultaneous stimulation of two sites, cerebellar and prefrontal for the combined tDCS condition) and considering a maximum of 4 mA injected current with high-density electrode montages (current density up to 0.63 mA/cm^2), the optimization algorithm generated assuming a left hemisphere stroke (right ipsi-DLPFC and left contra-CEREB stimulation), suggested 0.25 V/m simultaneously to both targets. This intensity was also retained to avoid possible side effects and warrant the safety/tolerability of our electrode montage. The "flipped version" of the former montage, hence assuming a right hemisphere stroke (right ipsi-DLPFC and the left contra-CEREB stimulation) was also simulated and delivered the same optimized electrode solution (see Fig. 2 for details).

Stimulation procedures

HD-tDCS will be delivered to all experimental groups with a Starstim-8® device, a wireless hybrid EEG/tES 8-channel system (Neuroelectrics, Barcelona, Spain). The Neuroelectrics Instrument Controller® software (NIC) associated with this hardware will be used to preprogram all HD-tDCS and EEG protocols and carry out interventions. Given the four-parallel arms of our study design (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC + contra-CEREB, and SHAM) and considering participants with left or right hemisphere stroke, a total of 8 HD-tDCS protocols will be pre-programmed.

During stimulation sessions, participants will wear a neoprene cap adapted to the circumference of their head ensuring correct placement for NG pistim® electrodes (πcm^2 surface, Ag/AgCl) embedded in SignaGel® (Parker laboratories, USA) to keep impedances below 10 KΩ. Every participant will receive 10sessions of tDCS, 20 min each and 5 daily sessions per week (Monday to Friday) during 2 consecutive weeks (W1 and W2 of participants' schedule).

The tDCS device integrates a maximum of 8 electrodes to deliver stimulation. However, note that depending on the experimental tDCS group, different electrodes will

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be actively involved in stimulation at each condition (see tDCS montage protocol section). To conceal the stimulation condition and preserve the blinding of the operator and the participant, all 8 available electrodes will always be positioned on the participant's head cap and will have their impedance tested. Crucial values such as mean voltage, current intensity, and impedance of the different electrodes employed will be recorded automatically during the sessions. Targeted regions are represented in Figs. 2 and 3.

Active tDCS conditions

Once the head cap is positioned and all the necessary electrodes are attached, the current intensity will be linearly increased (ramp up) for 30 secs to reach the peak current intensities delivered in each specific condition: (1) 1.736 mA intensity (0.55 mA/cm^2 density) in the ipsi-DLPFC group (receiving ipsilesional prefrontal stimulation); (2) 1.999 mA intensity (0.63 mA/cm^2 density) in the contra-CEREB group (receiving contralesional anterior cerebellar lobe stimulation); and (3) 3.735 mA intensity (0.55 mA/cm^2 density in the ipsi-DLPFC area and 0.63 mA/cm^2 in contra-CEREB) in the ipsi-DLPFC + contra-CEREB condition (receiving simultaneously dorsolateral prefrontal and cerebellar stimulation). Once the predefined intensity is reached, the current will be kept active for 20 min, a treatment duration that has been proven effective in prior studies with neurological patients [14, 15]. Finally, tDCS current will be ramped down for 30 secs at the end of the stimulation session.

During the delivery of tDCS, patients will be seated in a comfortable chair. To keep patients awake and restrict interindividual and intraindividual variability caused by a diversity of neural states present across subjects and sessions, participants will be asked to perform a simple computer-based game on a monitor placed 57 cm in front of them (hence at arm's reach) requiring keyboard presses (space bar) with their non-impaired hand, every time a moving dot contacts the borders of a centered rectangular placeholder (8 × 13 degrees of angle/cm).

Sham tDCS condition

The sham tDCS intervention will follow the same procedure as the active conditions, even though placebo current stimulation will be applied. At the beginning of the session, during the first 30 secs, electrical current will be ramped up emulating the ipsi-DLPFC + contra-CEREB stimulation condition. Immediately thereafter, a 5-secs ramp-down will cease the delivery of electrical current. During the following 20 min of the session no electrical current will be administered. At the end of the session, the same procedure will be repeated, delivering a 5-secs ramp up followed by a 30-secs ramp down to null intensity. This protocol commonly known as "FISSFO" (Fade In of Stimulation, brief real Stimulation, Fade Out) has been extensively used in clinical trials to mimic during sham conditions the tingling and itching skin sensations perceived during the ramp up and ramp down of tDCS intensity [45]. In the current clinical trial, patients will undergo tDCS treatment during the late subacute to chronic phase following a stroke, hence optimal for non-invasive neuromodulation, but during which, instances of spontaneous recovery might still operate. Moreover, for ethical reasons, while participating in our study, patients will continue their conventional physical rehabilitation program (see next section for details), which *per se* could also contribute recovery. In such circumstances, and also to counter improvement effects caused by familiarization and training in evaluation tasks performed several times during the study follow-up, a sham stimulation group is key to identify genuine recovery fostered by transcranial stimulation.

(See figure on next page.)

Fig. 2 Computational biophysical models of an in-house ipsilateral dorsolateral prefrontal (ipsi-DLPFC), contralateral anterior cerebellar lobe (contra-CEREB), and combined prefronto-cerebellar (ipsi-DLPFC + contra-CEREB) tDCS montages were developed in SimNIBS 3.2.3 using a 3D reconstruction of the MNI152 model brain. Electrode positions surrounded by red circles represent active anodes; those surrounded by blue circles, correspond to active cathodes; finally, electrodes surrounded by black circles represent inactive electrodes (placed in the head cap during the stimulation only to ensure the operator's blinding). **A** Optimized montage solution for the ipsilesional prefrontal target (DLPFC) assuming a left hemisphere stroke. Ag/AgCl πcm^2 electrode position and current intensities are defined by an optimization procedure resulting in the following scalp montage and currents to be delivered (10/20 EEG system): F3 (1.736 mA), F1 (− 1.222 mA), and FC5 (− 0.536 mA). **B** Illustration of the optimized solution for the contralesional anterior cerebellar lobe (CEREB) target assuming a left hemisphere stroke. Ag/AgCl πcm^2 electrode position on the scalp and intensities were automatically defined by an optimization procedure resulting in the following montage (10/20 EEG system): PO10 (1.960 mA), FT10 (− 0.550 mA), CP6 (− 0.180 mA), Oz (− 0.630 mA), and P9 (− 0.631 mA). **C** Final simulation merging DLPFC and cerebellar electrode montages assuming in this specific case a left hemisphere stroke, pertaining to the prefronto-cerebellar stimulated group. All images are presented in terms of total electric field strength (|E|). The visual color impact scale was normalized in all images from a minimum of 0 V/m (blue areas) up to a maximum of 0.36 V/m (red areas). Contra-CEREB: anodal contralesional cerebellum, ipsi-DLPFC: anodal ipsilesional dorsolateral prefrontal cortex, ipsi-DLPFC + contra-CEREB: anodal ipsilesional dorsolateral prefrontal cortex with anodal contralesional cerebellum, normE: electric field strength (|E|)

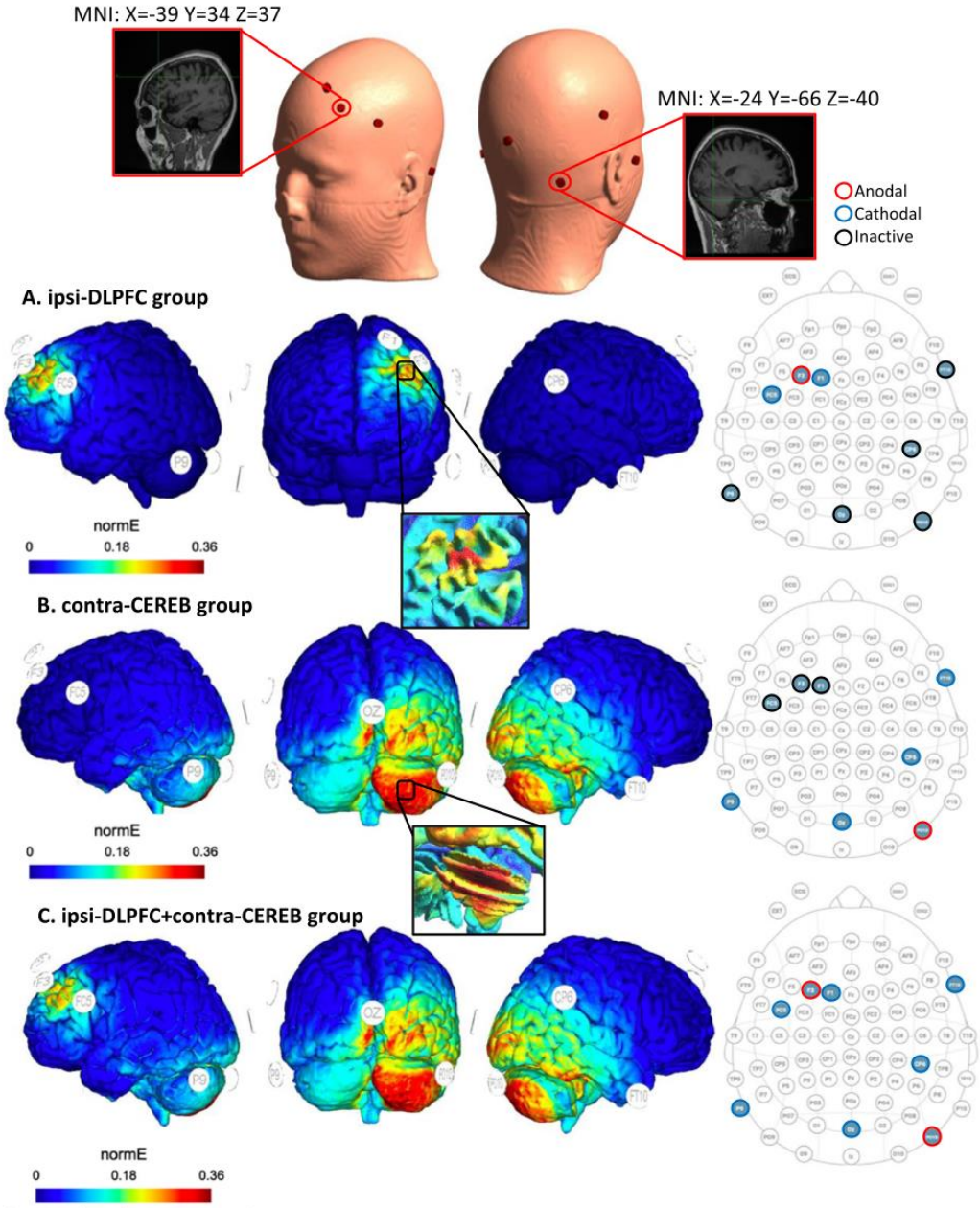


Fig. 2 (See legend on previous page.)

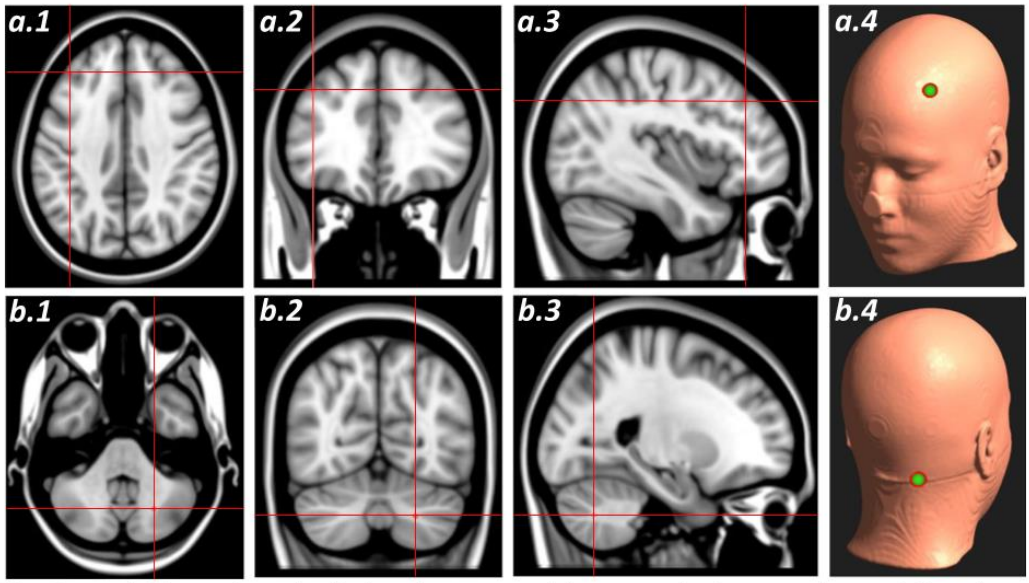


Fig. 3 Transcranial tDCS targeted regions (used for electrode optimization and subsequent stimulation) assuming in the image a left hemisphere stroke. Established MNI (Montreal Neurological Institute) coordinates have been selected ensuring a constant electric field impact (0.25 mA) in Brodmann area 46 for the dorsolateral prefrontal cortex (DLPFC) and lobules I–IV of theanterior cerebellar lobe (CEREB). **A** DLPFC target area (MNI coordinates $X = -39$, $Y = 34$, $Z = 37$) in an axial (*a.1*), coronal (*a.2*) sagittal (*a.3*) MRI sections and their scalp projection (*a.4*) views. **B** Cerebellar target area (MNI coordinates: $X = 24$, $Y = -66$, $Z = 40$) in axial (*b.1*), coronal (*b.2*), sagittal (*b.3*) MRI sections and their scalp projection (*b.4*) views. Target areas are represented in the MNI152 and labelled on a standard MRI model by means of an MRI-based frameless stereotaxic neuronavigation system (Brainsight)

Associated clinical rehabilitation

In order not to undermine optimal recovery potential and since we are testing an innovative tDCS protocol for which clinical efficacy is not warranted, all participants will be enrolled in parallel, in a similar "live" on-site rehabilitation program. Stimulation sessions will be conducted in the morning (8–12 am) and "live" rehabilitation sessions delivered independently in the same institution will follow. Rehabilitation activities during participation in the trial aim to maximize the chances of optimal recovery given the uncertain therapeutic value of our intervention and were specifically requested by our local Institutional Review Board for ethical reasons.

The on-site "live" program is based on an intense multidisciplinary rehabilitation plan encompassing specific goal-directed qualitative interventions, combining physical therapy, occupational therapy, neuropsychological, and speech therapy interventions, with a duration and periodicity of 1 to 2.5 hours a day, 2–3 days a week. Hence, all participants will conduct equivalent rehabilitation activities based on identical goal-directed qualitative occupational principles. On stimulation days, when hospital rehabilitation cannot be performed live on-site, participants will be instructed to carry out 1–2 hours of rehabilitation at home based on the same activities usually performed in the hospital. Clinical physical therapy is mainly focused on gross motor functions and postural control training (i.e., reaching, straightening, and support abilities), somatosensory integration, and gait rehabilitation. Occupational therapy exercise upper limb fine motor function, spasticity reduction, manual skill training, and multisensory stimulation. Finally, neuropsychological rehabilitation is mainly centered on training executive function and the management of emotions (i.e., impulsivity, liability, childish behavior, apathy, orientation and depression).

Safety monitoring

No major side effects of stimulation or the follow-up of stroke patients are to be expected given the well-known safety profile of tDCS interventions in neuropsychiatric patients. Nonetheless, in order to monitor safety and to evaluate tolerance and comfort to tDCS, participants will complete before and immediately following each stimulation session, a standardized adverse effect questionnaire [10] documenting the incidence and intensity of

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the most common side effects of tDCS stimulation (notably itching, pain, burning, fatigue and headache). Even if extremely unlikely, in case of a major adverse event-related or unrelated to stimulation such as for example a second stroke or an epileptic seizure participants will cease participation in the study and will be immediately provided with medical assistance and follow-up care in our institution.

Outcome measures

In agreement with our objectives, the primary outcome measure (addressing the main and key objective) of our trial will evaluate changes in the Fulg-Meyer Assessment (FMA) for the impaired upper limb. A set of secondary outcome measures recorded prior to and following tDCS treatment (addressing study objectives 2, 3, and 4) will assess respectively: (2.1) Patient's performance in a series of computer-based behavioral tasks evaluating visuo-motor adaptation (anterior cerebellar lobe contributions) and also sustained attention and cognitive control (prefrontal contributions); (2.2) Changes in resting-state and task-evoked EEG recordings and (2.3) set of clinical scales evaluating global stroke severity, cognitive impairment and their recovery, and correlations with predicted electric field distribution model features and stroke lesion hallmarks revealed by structural MRI neuroimaging. Find below a detailed explanation of the different outcome measures employed in our study.

Fugl-Meyer Assessment (FMA)

Addressing the primary or key goal of our study, the Fugl-Meyer Assessment (FMA) will be used to evaluate upper limb motor improvements. This is a widely employed tool used to assess motor impairment in post-stroke participants and considered one of the most comprehensive and reliable quantitative measures for motor hemiplegic dysfunction [46]. Among the sub-sections of this assessment we will focus on evaluating the upper limb/extremity motor domain (FMA-UE). Even so, the lower limb/extremity section of the assessment (FMA-LE) will be also administrated to evaluate the status and changes in lower limb motor function. The FMA-UE and FMA-LE include a series of items measuring movement, coordination and reflexes, each one scored on a 3-point ordinal scale (0 = cannot perform, 1 = performs partially, 2 = performs fully), with a total score of 0 points equaling absolute hemiplegia, and 100 points signalling sound motor function; of these 100 points, 66 are attributed to the upper extremity (FMA-UE) and 34 to the lower extremity (FMA-LE). For intra-subject pre-post-intervention assessments, an improvement greater than 6 points is usually defined as clinically significant.

Visuo-motor adaptation task

A visuo-motor adaptation task known to assess contributions from the anterior cerebellar lobe to voluntary motor function [47–50] programmed in a MATLAB environment (R2019a, Mathworks, USA) and Psycho-Toolbox will be implemented. During the task, participants will seat in a comfortable chair in an isolated room with no distractions at a distance of 57 cm (arm's reach) in front of a 15-inch computer monitor, holding a hand-joystick with their impaired paretic upper limb. Hand splints to ensure correct attachment to the joystick will be employed if necessary for all subsequent measures to keep conditions constant. During the task, the joystick position will be displayed as a red dot of 1-cm diameter. Participants will be asked to complete a series of consecutive trials to direct the cursor (a red dot) from the center of the screen (starting point) towards the interior of a randomly allocated peripheral target displayed as a green circle. Each trial will start with the green circle and the red dot in the center of the screen. Subsequently, additional green circles (targets) will appear randomly in 4 possible locations, equally spaced around a virtual circle respecting a homogeneous 5 cm distance from the starting point. Participants will have 10 secs to move the red dot and place it inside the circle and maintain such position for 0.5 s. Once completed, the green circle will jump back to the starting point, and participants had to replace the dot in the starting position to complete the trial. During the inter-trial interval (1.5 secs), a full gray screen with a center stimulus ("+") will be displayed to keep participants alert and ready for a subsequent trial. If the trial is not successfully completed, hence the participant does not succeed in placing the dot inside the circle during the allotted time window, the circle will automatically jump to the starting point. Visual feedback of the dot and the circles will be displayed in real time (see Fig. 4A). The task will be divided into 2 consecutive blocks. The first block (familiarization) will consist of 32 trials, and will be followed by a second block (motor adaptation)including 64 trials. During the familiarization block, trials will be conducted as previously described. During the motor adaptation block, a "force-field-like" perturbation will be implemented by introducing a constant 45° angular rotation between the trajectory of the red dot trajectory and the real movement of the joystick, deviating the trajectory of the former and forcing participants to compensate for such shifts in order to reach the target in the alloted time, hence engaging visuo-motor adaptation (learning) skills.

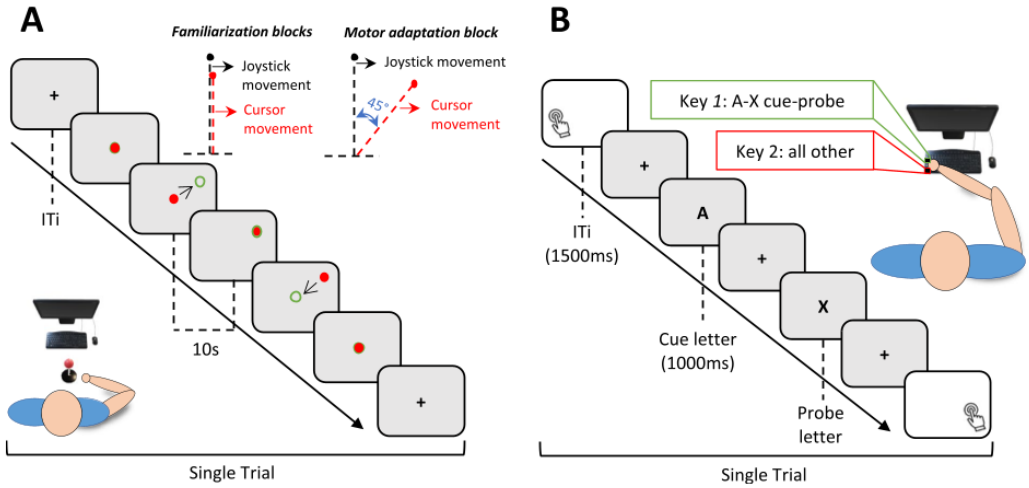


Fig. 4 Computer-based selected tasks to assess cerebellar (motor learning and adaptation) and dorsolateral prefrontal (sustained attention and cognitive control) contributions to motor functions and their changes following stimulation. **A** Visuo-motor adaptation experimental paradigm design and setup. Following a stimulus ("+"), a red cursor representing the joystick position is displayed in the center of a computer screen. Participants are required to direct the cursor moving the joystick towards a randomly allocated target (within a time window of 10 secs) displayed as a green circle among other targets, equally spaced around a virtual circle. In the motor adaptation block, a force field with a 45° constant perturbation is applied deviating the trajectory of the cursor respect the actual joystick trajectory, hence forcing the participant to adapt and learn how to compensate such a deviation. **B** AX-CPT experimental design. Participants are instructed to attend to serial presentation of letters and to make a target response each time the correct 'cue-probe' (A + X) combination is presented (by pressing the key "1" on a keyboard), or execute an alternative response (by pressig the key "z") for all other incorrect 'cue-probe' combinations (e.g., A + S, M + X). During the experimental recording, a total of 150 trials encompassing the presentation of a cue stimulus (1000 ms), a fixation stimulus "+" (1000 ms), and a probe stimulus (1000 ms) followed by an inter-trial interval (1500 ms) are completed. ITI: Inter-trial Interval

AX continuous performance task

The AX continuous performance task (AX-CPT) is a computer-based paradigm running in E-prime software (E-Prime®, Psychology Software Tools, Sharpsburg, PA, USA) which has been extensively used to explore DLPFC contributions to sustained attention and cognitive control subtended by prefrontal systems. Moreover, performance in the task has been correlated with the severity of the motor impairment [51–53]. In this paradigm, participants will be comfortably seated at a distance of 57 cm from a 15-inch computer screen in an isolated room with no distractions, and required to attend to a serial presentation of letters and provide a response (press the "Q" key on the keyboard) every time the cue-probe letter combination "A" + "X" is presented. Likewise, participants are also instructed to execute an alternative response (press the "Z" key) when any cue-probe letter combination other than "A" + "X" is displayed on the screen (e.g., "A" + "S"). Participants are required to respond as quickly and accurately as possible after each cue-probe combination during a response interval using their non-impaired hand (please see Fig. 4B). Each trial consists of a cue letter stimulus (1000 ms duration), the "+" fixation stimulus (1000 ms duration), and a probe letter stimulus (1000 ms duration) followed by a 1500-ms response interval displaying a white screen. Letters on the screen will always be displayed in Times font (40 size), in black capital letters on a white background. The task will be split into two parts: first, (1) a series of practice trials acclimating the participant to the paradigm while receiving the researcher's constant feedback to ensure participants correctly understand the task; second, (2) the experimental trials will be launched after a short rest (duration determine ad libitum by subject preferences). Twelve trials will be presented during a practice block followed by an experimental block including a total of 150 trials. Responses in both the practice and the experimental blocks will be recorded, but only the latter will be used for statistical analyses.

EEG acquisition

EEG data will be recorded with the same HD-tDCS equipment used for stimulation, a Starstim® 8 channel device controlled by the Neuroelectrics Instrument Controller® software (Neuroelectrics, Barcelona,

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Spain) able to sample scalp EEG signals at a frequency of 500 Hz using scalp NG pistim® electrodes (πcm^2 Ag/AgCl) and SignaGel® (Parker laboratories, USA). Skin/electrode impedance values will be automatically monitored and kept at all times below 5 KΩ. The 8 recording electrodes will be distributed across left and right hemi scalp positions (F3, F4, C3, C4, P3, P4, PO10, and PO9) according to the 10/20 EEG system, to capture among other sources, derived EEG activity associated to prefronto-central, and fronto-parietal motor/premotor networks. All EEG sessions will be conducted under the same conditions with a combined ground reference placed in the right earlobe. Ten minutes of resting-state EEG data (eyes open fixating on a target located at arm's reach, ~ 57 cm) will be acquired during the baseline assessment, the post-stimulation regime assessment, and during the follow-up visit. EEG will be also continuously recorded during our visuo-motor adaptation task assessing cerebellar contributions to motor learning and during the AX-CPT paradigm assessing dorsolateral prefrontal contributions to sustained attention and cognitive control. Finally, 5 min of continuous resting-state EEG data (eyes open) will also be recorded prior to and following each of the 10 sessions of tDCS stimulation.

National institutes of health stroke scale

The National Institutes of Health Stroke Scale (NIHSS) is a quantitative scale of stroke-related neurologic deficits widely used to characterize baseline impairment in clinical trials [54]. It explores consciousness level, visual field surface, language function, the presence of hemineglect, hemiplegia, movement disorders, and impairments of sensory function. It consists of 15 items, each one scored from 0 to 4, to reach a total of 42 potential points, in which higher scores signal more severe impairment (0 = no stroke symptoms, 1–4 = minor impairment, 5–15 = moderate impairment, 16–20 = moderate to severe impairment, 21–42 = severe impairment). We will use the NIHSS to characterize the stroke severity of our patients and also use it to verify the comparability of each of the 4 experimental tDCS groups defined in our trial.

The Montreal cognitive assessment

The Montreal Cognitive Assessment (MoCA) is a quantitative screening tool used to explore stroke-related cognitive deficits [55]. It is made of a series of questions and tasks specifically designed to assess visuo-spatial and executive function, working memory, short-term memory, sustained attention, abstraction, language, and orientation in time and space. It contains a total of 10 items, for a total of 30 points, and lower scores indicate higher levels of impairment (26–30 = no stroke cognitive impairment, 18–25 = mild cognitive impairment, 10–17 = moderate cognitive impairment, 0–10 = severe cognitive impairment). We will use the MoCA to evaluate participants' cognitive impairment/improvement and also to asses group comparability.

Screening of hemispatial neglect

In order to monitor the presence of hemispatial neglect, participants will conduct a letter cancellation test, a Bells cancellation test, and a line bisection test. The letter cancellation test quantifies the presence of visuo-spatial neglect and visual search/scanning deficits [56]. In the task, a total of n letters is distributed into 6 lines presented in paper format. Among them, the letter "H" is repeated 104 times. Participants will be asked to visually screen the paper sheet and find and outline as many letters "H" as they can find. The Bells cancellation test quantifies visual neglect deficits in the extra personal space [57]. A total of 35 bells are embedded within 280 distractors presented in paper format. Participants will be instructed to circle or cancel off all drawings corresponding to bells. All stimuli are of the same size and displayed in black color over a white background. Though they might appear randomly distributed, bells are presented in 7 columns; 3 columns on the left and the right hemifields and one in the middle, with 5 bells and 40 distractors on each one. The sheet of paper with the test display will be placed right in the middle of the visual field and the participant is free to explore the document with his/her gaze. Finally, the line bisection test identifies and quantifies visuo-spatial orienting impairments and spatial neglect deficits [58]. In this task, several examples of two types of black lines, 5 and 10 cm long, are presented on a white paper sheet placed in the middle of the participant's visual field. Participants, who are free to explore stimuli with their gaze, are instructed to identify the center of each horizontal line and bisect it with a pencil.

MRI imaging acquisition

MRI structural scans will be obtained from all stroke patients. These will be either drawn in anonymized coded form from recent recordings stored as clinical records by an authorized neurologist working for the protocol, or if not available, acquired de novo within the 2 weeks before tDCS treatment onset. A 3-Tesla MRI scanner (Siemens Healthcare, Germany) located at the neuroradiology department at Hospital Universitari Joan XXIII in Tarragona will be used for image acquisition. In that case, a T1-weighted 3D anatomical sequence will be carried out with the following parameters: repetition time = 2500 ms, echo-time = 2.12 ms, number of slices = 156, slice thickness = 0.94 mm, matrix size = 232 × 288, in-plane resolution = 0.83 mm × 0.83 mm, and flip angle = 9°.

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Data collection and management

All data generated by the study will be hosted in a secured internal server, property of the *Universitat Rovira i Virgili* (URV) in Tarragona. A shared encrypted database allowing access to anonymized datasets only to authorized co-investigators with a valid personal ID has been set up.Our clinical trial will generate three large classes of records: (1) demographic information, (2) clinical outcome datasets, and (3) neuroimaging and electrophysiological datasets including MRI scans and EEG recordings. Two computers, property of the URV, secured with personal encrypted passwords, will be used for databasing. Given the volume and complexity of the pre-processing involved in the analysis of MRI and EEG datasets, these datasets will be stored in specific computers handled by expert investigators associated to the project. To ensure data quality, all co-investigators and associated personnel taking part in the E-Brain trial will be trained by the principal investigators (co-authors XC-T, MT-C, and AV-C) to perform any of its procedures (screening, recruitment, informed consent, randomization and allocation, blinding, cognitive and clinical evaluations, EEG/MRI recordings, and tDCS application) by following strictly the conditions, criteria, materials, and methods established in the written protocol approved by our local IRB. In the same vein, all data will be collected by the same institution, and co-investigators will be specifically trained to strictly respect and implement established protocols for data collection, anonymization, storage, analytical strategies and "cloud" data management. To maintain at all times data anonymity and confidentiality, a reference number representing the participant until the study is terminated identifying individual datasets in our databases will be assigned to each participant at inclusion. Only co-authors XC-T, MT-C, and AV-C will be provided access to the full dataset generated by the study, with the exception of group allocation information which, as stated previously, will only be unblinded to these investigators, once data collection and analyses are completed.

Data analyses

Analysis of clinical and cognitive performance outcome measures

Outcomes extracted from computer-based tasks and clinical scores (see the previous section) will be analyzed to explore the differential ability of active tDCS conditions compared to sham stimulation to induce upper limb motor recovery following a stroke. For the clinical tests (FMA, NIHSS, MoCA, and hemispatial neglect tests), mean group scores post vs. pre-tDCS baseline intervention,and during the follow-up assessment, will be compared across experimental groups. Sociodemographic data will also be compared between treatment groups (Group 1: ipsi-DLPFC, Group 2: contra-CEREB, Group 3: ipsi-DLPFC + contra-CEREB, Group 4: SHAM) to identify potential collapse factors.

In order to quantify changes in motor performance with the visuo-motor adaptation task,and assess tDCS effects applied over the cerebellum or the dorsolateral prefrontal areas on upper limb voluntary motion, the following set of measures will be used: (i) the angular trajectory error ("SumErr"; cm) estimating accuracy, consisting of the sum of observed deviations from the ideal linear trajectory at peak tangential velocity (PV)for each trial of the motor adaptation block; (ii) the area under the learning curve (AUC) measuring patients' adaptation rate,calculated by performing a power fit on the mean learning values of each participant; (iii) the coefficient of variation (CV)measuring the variability of motor performance during the adaptation plateau phase (i.e., when the AUC is stabilized and reaches stable levels) and calculated as the standard deviation (SD) divided by the mean over trials belonging to the plateau phase (AUC/Time-to-target). All these metrics will be represented in ordinal and mean group scores for post vs. pre-tDCS baseline intervention, and for the follow-up milestone and compared across tDCS groups.

In order to quantify sustained attentional abilities (as a proxy of dorsolateral prefrontal modulation by tDCS) with the AX-CPT task, we will analyse the total number of errors used as a proxy of sustained attention failure. An ordinal value will be taken and group means will be compared between pre- vs. post-tDCS and for the follow-up milestone. In order to indentify and quantify changes of visuo-spatial performance (as a proxy of visuo-spatial orienting and selective attentional disability), the number of crossed/circled letters (letter cancellation test) or bells (Bells cancellation test) and the shift (in mm and % of the total line length) of manual bisections from the precise midpoint of each line (line bisection test) will be analysed. Mean group total ordinal scores will be compared between groups acrossthe different time points (pre-, post-tDCS s and follow-up milestones). Other unanticipated complementary metrics could also be taken into account for the final analyses.

EEG data analysis

EEG data will be preprocessed using a hierarchically organized pipeline proceeding as follows [59]. Data will be first segmented into contiguous epochs after a preliminary investigation on which epoch length is the most robust for analyses. Then, a 2nd-order infinite Butterworth forward and backward filter with a 0.5–45 Hz low-pass high-pass filter at a resolution of 2 Hz (500 ms time windows) with a Hanning taper window will be applied. Eye movements or muscle-related artifacts will

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be removed using a common Independent Component Analysis (ICA), supervised and manually corrected after expert visual verification. Epochs with signals exceeding 120μv peak-to-peak amplitude will be also removed. Finally, channels will be re-referenced to their common average. Once data pre-processing is completed, (i) power spectrum density (PSD), (ii) connectivity, and (iii) complexity measures will be calculated. These approaches will be individually conducted on each subject for every condition or task (resting-state recordings, visuo-motor adaptation task, AX-CPT task, associated to everytime point of our study timeline (baseline, post-tDCS, follow-up).

A time-frequency analysis will be conducted in order to explore pre tDCS (baseline) vs. post tDCS and vs. follow-up tDCS-related modulations of patients' oscillatory activity. To this end, the amplitude of the power spectrum density (μV^2) for each frequency band (δ: 0.5–4 Hz; θ: 4–8 Hz; α: 8–12 Hz; β: 12–30 Hz; γ: 30–60 Hz) will be computed by means of a Fast Fourier Transformation (FFT) using Welch's method with a 500-ms periodogram. Relative and absolute spectral normalized power (NP) will be then extracted for each EEG electrode. Normalized average power for all frequency bands will be computed and plotted to explore differences between pre-tDCS (baseline) vs. post-tDCS and vs. follow-up milestones within each group and to compare differencesacross independent groups stimulated with different tDCS approaches.

Functional connectivity analyses by means of EEG sensor level coherence measures will be conducted to explore the "reshaping" of intra-hemispheric and inter-hemispheric connectivity (assessing functional integration and segregation mechanisms at the network level) by tDCS approaches. Given that resting-state data can be influenced by false positive correlations and to avoid the impact of volume conduction on raw coherence measures, we will compute more specifically the imaginary part of coherence (ImCoh) [60] by means of a Fast Fourier Transformation (FFT). Intra-hemispheric functional connectivity analysis will focus on synchronization measures between ipsilesional DLPFC and M1/premotor (electrodes and F3-C3/F4-C4) and also ipsilesional DLPFC and parietal systems (electrodes F3-P3/F4-P4). For inter-hemispheric functional connectivity, our analyses will focus on synchronization measures between premotor/M1 (electrodes C3–C4) and also prefrontal systems (electrodes F3–F4). Overall, an 8×8 connectivity matrix contemplating all connections between all possible electrode combinations, will be explored.

Complexity analyses will explore tDCS-related changes of neuroplastic properties and estimate the predictability of EEG signals following a given tDCS condition by calculating multiscale sample entropy (MSE). This estimate quantifies the probability that neighboring data points may be in a predetermined range in a time series $\{x_1, x_2, ...x_N\}$, computing how often patterns reoccur in a time-domain sample (i.e., temporal irregularity prediction in the time-domain). The computation of MSE is divided into two steps: (1) first, a moving-averaging procedure is computed to express the dynamic representation of a system; (2) Second, the degree of predictability is measured for each of the moving-averaged time series z^r by means of the sample entropy ($Sample_{En}$) method (see [61] for a complete formulation of MSE).

MRI data analysis

MRI T1 sequence will be used to characterize stroke lesion features. To this end, a lesion overlay in normalized space for the complete sample of MCA stroke participants will be compiled. First, brain lesions will be manually drawn outlining damaged areas directly on the T1-weighted MRI sequences in native space using MRIcron software (v1.0.2.), outlining the precise anatomical boundaries of the stroke lesion. A graphic tablet (WACOM One) will be used for lesion mask delineation by an expert researcher (co-author XC-T) trained in neuroimaging and neuroanatomy upon advice from additional co-investigators (co-authors AV-C, MT, and MTC). Then, lesion masks will be normalized in SPM12 using a unified segmentation method depicting co-registered image lesions overlapped in a template atlas. Further, according to parcellated cortical structures based on Brodmann areas, the percentage of impacted voxels within each parcel and damaged tracts will be characterized and correlated with the set of above-mentioned outcome measures.

Biophysical E-field models

As indicated above, electrode placement for the different tDCS conditions of our trial has been optimized by using a biophysical model of current distribution on a standard head/brain volume (software SimNIBS 3.2.3). Nonetheless, due to anatomical interindividual differences, the impact of electric currents at targeted structures (the DLPFC and the anterior lobe of the cerebellum) can slightly differ from subject to subject. For that reason, individual biophysical models will be generated to estimate the E-field (electrical current fields) strength distribution on each participant's MRI. On such basis, we will study *post hoc* the influence of model-generated variables (peak E-field strength at target, and the volume of anatomical layers the field needs to go through to reach the target) and their association with motor clinical recovery outcomes, the modulation of prefrontal (DLPFC) or cerebellar (CEREB) cognitive contributions and EEG

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outcome measures. To this end, individual T1-weighted MRI head models will be reconstructed for each patient. Tisue segmentation layers (air, bone, CSF, eyes, GM, WM, and skin) will be manually corrected with a graphic tablet by an experienced researcher (co-author XC-T) upon advice by expert co-investigator (co-authors AV-C and MT). After manual correction, head models will be re-reconstructed to ensure acceptable delineation of lesion and tissue boundaries. Then, tDCS current distribution and target peak E-field strength induced by the aforementioned electrode montages will be simulated using finite-element modeling (FEM) solving Laplace equation. To perform further voxel-level analysis correlations, individual E-field distribution will be normalized and its magnitude co-registered into a standard head model. Data extracted from individual models will be correlated to modeled peak currents on stimulated sites for each participant (ipsilesional DLPFC, contralesional CEREB, or the combination thereof) with the magnitude of motor recovery, cognitive modulations in dorsolateral prefrontal and cerebellar modulated cognitive outcomes, anatomical and lesion features. Additionally, we will correlate peak current density at cortical targets modelled individually with resting-state EEG measures.

Statistical analyses

In order to evaluate the therapeutic potential of each tDCS intervention in post-stroke motor recovery, we will first asses the data distribution of the Fugl-Meyer Assessment by means of Shapiro–Wilk method. In the case of normal distribution, clinical outcomes will be compared using repeated measures analysis of variance (two-way ANOVA) with "TIME" (baseline, post-tDCS, follow-up) as within-subject factor, and "GROUP" (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC + contra-CEREB, SHAM) as between-subject factor. Post hoc pair-wise comparisons will be performed using a Bonferroni correction for multiple comparisons. In the case of non-normal data distribution, a non-parametric Kruskal–Wallis test will replace repeated measures ANOVA. Linear mixed models and Pearson's (for normally distributed data) or Spearman's correlation coefficients corrected for multiple comparisons between AX-CPT, visuo-motor adaptation task, and the Fugl-Meyer outcomes will be used to explore behavioral correlation between motor, attentional, and cognitive control domains. All data will be presented as mean ± standard deviation and statistical significance will be set for all tests at $p \leq 0.05$.

In order to characterize the electrophysiological EEG effects induced by our interventions, we will first explore correlations with behavioral data and biomarkers of recovery. First, power spectral density (PSD) changes for each frequency band will be compared between stimulation groups using two-tailed ANOVA ($P = 0.05$), with "TIME" as the within-subject factor and "GROUP" as the between-subject factor. To correct for multiple comparisons, non-parametric cluster-based permutation statistics with Montecarlo sampling (1000 permutations) will be applied, allowing the examination of global effects across all electrodes while controlling for multiple comparisons at the sensor level, without the need of prior assumptions about effect location. Finally, connectivity (imaginary part of coherence measures) and entropy effects (complexity of temporal dynamics) of the electrophysiological response will be also explored. The same statistical procedure as for PSD exploration applying repeated measures ANOVA with non-parametric cluster-based permutation statistics and MonteCarlo sampling (1000 permutation) will be used, with "TIME" as within factor, and "GROUP" as between factor. Individually averaged connectivity and entropy values (total averages) and individual topographical maps at the electrode level will be examined to detect specific regions sensible to the tDCS intervention.

The individual differences across the study follow-up for the Fugl-Meyer Assessment evaluating upper/lower limb motor recovery,visuo-motor adaptation task outcomes assessing cerebellar contributions to motor learning and the AX-CPT task assessing sustained attention and cognitive control will be correlated (Pearson's or Spearman correlation coefficients, depending on data normality) with the individuals' electric fields voxel-by-voxel magnitude (an underneath cut-off of 0.25 V/m in total field strength will be positioned as a threshold in the power to induce neuronal effects). Moreover, the |E| and |nE| extracted values and the total injected current will be also correlated with the same outcome measures. Otherwise, E-field components will be correlated with specific EEG features reaching statistical significance in the power spectrum density (μV^2), connectivity, and complexity analyses. Finally, multivariate regression models will be computed to explore correlations between electrophysiological responses (μV^2, imaginary coherence and entropy measures), induced E-field, and behavioral outcomes.

All data will be analyzed according to the intention-to-treat principle. The data of all patients with complete datasets (at W0, W3, and W7 time points) will be analyzed according to their randomized tDCS group. All available data will be included in the analysis. Missing data will be imputed by means of a constrained longitudinal data analysis (cLDA) which will estimate unconditionally lost data.

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Discussion

The current study protocol (*E-Brain*) aims at assessing the immediate and longer-term clinical potential of ten accumulative sessions of anodal high-density tDCS in patients with upper limb motor disability during the chronic phase of a unilateral middle cerebral artery stroke. The novelty of our protocol is that instead of aiming to directly modulate damaged motor areas or their contralesional homologues, it focuses on assessing the isolated or combined stimulation of two regions such as the ipsilesional prefrontal cortex (DLPFC) and the contralateral anterior cerebellar lobe (CEREB), not directly involved (as premotor and primary motor systems do) in the execution of motor activity, but instead contributing indirectly to such via the modulation of associated cognitive processes.

A high number of prior conventional NIBS stimulation studies using repetitive TMS or tDCS in post-stroke motor dysfunctions have based their interventions on (i) the upregulation (with anodal tDCS, high-frequency rTMS or iTBS patterns) of ipsilesional M1/premotor systems; (ii) the downregulation (with cathodal tDCS, low-frequency rTMS, or cTBS patterns) of spared contralesional M1/premotor systems by virtue of the transcallosal rivalrous mutually inhibitory interactions, remapping, reorganizing, or normalizing abnormal excitability levels in lesional and perilesional areas [62]; or (iii) the upregulation of motor control/coordination systems such as the supplementary motor area (SMA) or the cerebellum. Unfortunately, initial enthusiasm for many of these approaches tested in small clinical trials has dwindled due to the lack of consistent effects when evaluated in larger populations of patients, and efficacy remains debated [63]. For this reason, it is paramount to explore and provide proof-of-concept for new treatments based on the manipulation of cortical sites, with the ability to drive improvements by acting on spared 'non-purely motor ' regions indirectly contributing to the recovery of voluntary motion via the modulation of associated cognitive processes and network-synchronization mechanisms.

In the cognitive domain, anodal tDCS over the ipsilesional DLPFC and/or cathodal stimulation of the contralesional DLPFC have shown efficacy in the modulation of prefrontal functions such as sustained attention and cognitive control [64–66]. Likewise, the modulation of the anterior lobe of the cerebellum has shown promise in stroke patients, given its active role in motor learning, and motor coordination, and its ability to contribute to motor "reorganization" when premotor or primary corticospinal systems are severely damaged [32–34]. Additionally, the neural signature of middle cerebral artery damage has revealed interactions between alterations of sustained attention deficits and impairments of motor function, emphasizing the importance of inter-areal communication in motor rehabilitation [20], hence the need for restorative stimulation methodologies able to integrate large-scale network-wide synchronization mechanisms across these structures. However, limitations in terms of spatial resolution posed by clinical EEG with few recording sensors-useful to explore the clinical evolution of neurophysiological markers in individual patients-has limited our comprehension of the underlying processes subtending motor recovery in stroke. Moreover, technical limitations of the first generation of tDCS devices to implement multi-site stimulation (i.e., targeting with acceptable spatial resolution via high-density montages different cortical nodes simultaneously) have contributed to keeping 'network' neuromodulation approaches poorly explored.

In such context, the protocol *E-Brain* aims at comparing in separate and independent patient groups three active anodal tDCS conditions (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC + contra-CEREB) and a SHAM tDCS intervention. Our design will contribute to identifying the most beneficial strategy comparing single-site (monofocal) or combined (bifocal) dual-site approaches by means of high-density tDCS. The study will also ascertain changes in DLPFC and anterior cerebellar lobe modulation by means of tasks specifically assessing sustained attention/cognitive control and visuo-motor adaptation skills respectively, to verify that upper limb motor improvements are mediated by the modulation of such contributing networks. Moreover, different EEG synchrony measures recorded along the intervention will report on local and "large-scale" modulations of primary motor systems from distant regions, via changes in functional connectivity and local and long-range synchronization mechanisms [67] and reveal the longer-term neuroplastic properties associated with such effects.

Still a key challenge for clinical neuroscience [68, 69], this approach is currently inspiring a transition from conventional "single-site" neuromodulation focused on targeting directly impaired systems, towards multifocal stimulation set-ups with multiple electrical sources able to address more holistically, network dysfunction [70, 71]. Moreover, it is supported for example by recent work demonstrating higher modulatory power on cortical reactivity [66] and changes in corticospinal excitability [72, 73] by dual-site (bifocal) stimulation as compared to single-site (monofocal) tDCS approaches. In this context, our protocol will be among the first fully adopting the notion of post-stroke motor paralysis as a network impairment involving not only motor but also non-motor cognitive systems contributing indirectly to voluntary motion. Under such perspective, we will pursue the

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modulation of network dysfunction via a circuit-based therapeutic approach and promote local and global synergistic network-wide effects ("reshaping") between non-purely primary motor regions (such as the prefrontal cortex and the anterior cerebellar lobe) and motor and premotor systems to facilitate motor post-stroke rehabilitation and effective recovery.

Our protocol is however not exempt from risk and might suffer from potential limitations, which we will try to minimize. First, recovery of lost motor function occurs slowly and gradually. Thus, the ten sessions of stimulation and a monthly follow-up planned for our patients might not be sufficient to drive or account for significant motor skill re-acquisition. Nonetheless, a more intense (i.e., a higher number of daily and cumulated sessions over time) and longer-lasting regime or follow-up period is currently outside of the scope of our protocol and limited by the scarcity of available time and resources. Two weeks of stimulation and a single monthly post-treatment follow-up seems a reasonable compromise for an experimental trial that once proven potentially successful, can be developed at a larger scale. Second, stroke patients even if sharing the same cardinal symptom, upper hand motor paralysis, can be differently affected by types of lesional mechanisms, lesion volume and lesion location, impacting the severity of the impairments, their ability to fully understand and perform motor or cognitive tasks hence compromise the reliability of behavioral and EEG evidence. Therefore, even if the *MinimPy* randomization algorithm counterbalancing groups by sex, age, and stroke type (ischemic/hemorrhagic) should minimize such risk, interindividual variability in lesion extent and clinical severity might be not equally distributed across the four experimental groups, precluding comparability. Third and least, the "live" on-site and at home rehabilitation activities associated to this protocol could slightly differ across patients, whereas uncontrollable factors such inner motivation or the intensity of unregulated outside activities with rehabilitative value could interfere and mask the real impact of tDCS interventions. For these reasons, on-site "live" rehabilitation programs during the treatment will be rightly monitored to make sure patients' programs are comparable across groups and kept unchanged. Additionally, patients will be regularly questioned with regard to their daily life activities during participation in the study and all their comments will be documented.

In sum, the alarming pandemic-like rates reached by the consequences of acquired brain damage in developed societies [74] and the limitations shown by conventional monofocal TMS or tDCS approaches in post-stroke motor rehabilitation calls for the design and assessment of novel treatments in experimental double-blind, controlled trials. Our protocol will test an innovative tDCS-based strategy, easy to implement clinically, aiming at modulating associated structures contributing respectively to sustained attention and cognitive control by dorsolateral prefrontal networks and also to visuo-motor adaptation by anterior lobe cerebellar regions. More generally, beyond pure clinical applications in motor improvement after an MCA stroke, our study is designed to provide insight on the anatomical and physiological foundations of motor impairments and tDCS neuroplastic phenomena driving recovery. Finally, if our intervention demonstrates clinical efficacy, our protocol will pave the way for the development of individually customized tDCS strategies based on multi-site interventions at a larger scale and the design of more sophisticated and better-adapted neuromodulation technologies.

Trial status

The first version of the study protocol was approved by the local ethics committee at the Institut d'Investigació Sanitària Pere Virgili (IISPV, Tarragona, Spain) on July 9th 2021. A pilot group of $n=8$ patients, which will not be included in the final analyses served to pilot the initial version of the protocol, hence to improve its design and content, and to test the adequacy, feasibility, and reliability of some of the assessment tasks, leading to the current final version. Participant recruitment began on March 2022; and since then, $n=11$ participants have successfully completed the protocol. Participant recruitment is planned to be completed by end of December 2025.

Abbreviations

AX-CPT	AX continuous performance task
BA	Brodmann area
CAT12	Computational anatomy toolbox 12
CEREB	Cerebellum
CNS	Central nervous system
Coords	Coordinates
CSF	Cerebrospinal fluid
contra-CEREB	Contralesional cerebellar stimulation
CT	Computed tomography
DAN	Dorsal attention network
DTI	Diffusion tensor imaging
DLPFC	Dorsolateral prefrontal cortex
EEG	Electroencephalography
E-field	Electric field
FMA	Fugl-Meyer assessment
fMRI	Functional magnetic resonance image
GM	Gray Matter
HD-tDCS	High-density transcranial direct current stimulation
Hz	Hertz
ipsi-DLPFC	Ipsilesional dorsolateral prefrontal cortex
mA	Milliamps
MCA	Middle cerebral artery
MNI	Montreal Neurological Institute
MoCA	Montreal cognitive assessment
MRI	Magnetic resonance imaging
NIBS	Non-invasive brain stimulation
NIHSS	National institute of health stroke scale

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BSIdir	Pair-wise derived brain symmetry index
PET	Positron emission tomography
PSD	Power spectrum density
ROIs	Regions of interest
SMN	Sensory-motor network
SPM12	Statistical parametric mapping
tDCS	Transcranial direct current stimulation
tES	Transcranial electric stimulation
V/m	Volts per meter
W	Week
WM	White Matter
|E|	Electric field strength
|nE|	Normal component of the electric field

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13063-023-07680-8.

Additional file 1. Standard protocol items: recommendation for interventional trials (SPIRIT) 2013 checklist: recommended items to address in a clinical trial protocol and related documents.

Additional file 2: Supplementary Table. World health organization trial registration data set.

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Dissemination policy
The findings of the current study will be published in international peer reviewed journals and presented at national and international scientific conferences. CONSORT guidelines will be followed at all times and co-authorship will comply with Vancouver rules. Anonymized datasets of primary or secondary outcome measures and computer scripts used for data analysis will be published in a online open-access repository if requested by the editor or alternatively they will be posted in our own institutional repository. Results will be also specifically communicated to participants and health professionals directly or indirectly involved or interested in the outcomes of our trial.

Authors' contributions
All authors read and approved the current manuscript. XC-T, MB, MTC, and AV-C wrote the first version of the manuscript. XC-T, AV-C, and MTC contributed to the conception and design of the clinical trial and will be in charge of its implementation and coordination. MF and RMS will be in charge of participant recruitment, randomization, and data collection (MRI and demographics). XC-T, MTC, MV-L, and AV-C will be in charge of data collection, analysis, and interpretation. XC-T, MF, RMS, and MV-L will be in charge of tDCS interventions and evaluations. MB, BB, and CG designed and adapted the cerebellar assessment computer paradigm and will contribute to analyses and its interpretation, whereas MT will be in charge of assisting with MRI-based lesion analyses. AV-C and MTC will supervise all aspects of the protocol and share senior authorship. The ethical committee of pharmacological research (CEIm) of the Institut d'investigació Sanitària Pere Virgili in Tarragona will act as the Data Monitoring Committee (DMC) of our study. In agreement with the CEIm, no interim analyses are planned. However, this independent instance will audit annually the progress made by the current clinical trial and authorize its continuation. Additional information on the rights and obligations of such a committee can be found at https://www.iispv.cat.

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Availability of data and materials
Not applicable. No data is available at this point because authors are still in the process of gathering such.

Declarations

Ethics approval and consent to participate
The protocol was approved the 27th of June 2021 (registration number: 077/2021, version: V.1_06/05/2021) by the local ethics committee at Institut d'Investigació Sanitària Pere Virgili (IISPV, Tarragona, Spain) and is in accordance with the Declaration of Helsinki and Spanish and EU regulations. All participants will be required to provide informed consent, which will be gathered by an authorized investigator of the study (XC-T, RMS, or MTC). The present study is registered on ClinicalTrials.gov (NCT05329818).

Consent for publication
EEG and MRI images or any other clinical details will not compromise participant anonymity.

Competing interests
The authors declare that they have no competing interests.

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4.3.2 Manuscript 7: Transcranial direct current stimulation on pre-frontal and/or cerebellar systems to improve motor function in stroke patients: Preliminary analyses from an ongoing pilot study

Transcranial direct current stimulation on pre-frontal and/or cerebellar systems to improve motor function in stroke patients: Preliminary analyses from an ongoing pilot study

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ABSTRACT

Middle cerebral artery strokes impact cognitive and motor function compromising human voluntary motion. Anodal transcranial electrical stimulation of the cerebellum has shown promise boosting and sculpting brain activity towards states favouring post-stroke motor-impairment recovery. We here investigated the effects of a transcranial direct current stimulation (tDCS) 10-day regime of cerebellar or prefrontal cortex stimulation (and both combined) on the recovery of motor function assessed by means of the Fugl-Meyer Assessment, different cognitive scores and computer-based visuo-motor adaptation tasks. A cohort of patients affected by middle cerebral artery stroke (n=27) at the subacute-to-chronic stage with motor deficits was randomized to one of 4 anodal tDCS stimulation conditions/Groups: Active Ipsilesional dorsolateral prefrontal cortex (DLPFC) stimulation (Group 1), Active Contralesional cerebellar stimulation (Group 2), Active Combined ipsilesional DLPFC and contralesional cerebellar stimulation (Group 3) and Sham tDCS stimulation (Group 4). As expected, clinical evaluations revealed an important degree of interindividual variability in terms of the magnitude of the motor deficits and their response to anodal tDCS treatment. The treatment was well tolerated by all participants, nonetheless, at this stage of recruitment, none of the tested active anodal tDCS conditions significantly improved motor function compared to sham. Interestingly, a non significant trend towards superior functional motor scores in patients undergoing either cerebellar or combined fronto-cerebellar simultaneous tDCS stimulation emerged and might deserve further attention as the recruitment is completed.

Keywords: Brain Stimulation, Stroke, Visuomotor Adaptation, Transcranial electrical stimulation, Cerebellum, Neurorehabilitation.

HIGHLIGHTS

1. Four different anodal Transcranial electrical stimulation strategies (including sham) delivered during 10 consecutive days were evaluated in MCA stroke subjects affected with different degrees of motor impairment.
2. Analyses reveals a high degree of interindividual variability with regards to the magnitude and type of motor deficits and cognitive evaluations and their response to the different stimulation conditions.
3. Clinically-based outcomes indicate superior functional improvements in subjects undergoing combined pre-fronto-cerebellar tDCS stimulation.
4. A non-significant pattern suggesting superior visuomotor adaptation performance after cerebellar tDCS, and increased variability in this same task following combined pre-fronto-cerebellar tDCS stimulation were noted.

INTRODUCTION

Middle cerebral artery stroke (MCA) is known to cause structural damage to frontal regions and pathways involved in the planning, control and execution of motor function. Additionally, either directly or via wallerian degeneration of white matter bundles, damage easily reaches further cortical and subcortical systems with a diversity of motor, sensory and cognitive contributions to the former. Consequently, an emerging set of motor impairments characterized by clinical scales or more sophisticated computer-based approaches must be understood as a combination of local and network effects which ultimately alter network communication and the integration of key feedback signals leading to optimized motion[1–3]. Relevantly, neuroimaging-based studies have associated MCA strokes and an abnormal recruitment of right lateralized fronto-parietal networks involved in attentional orienting in space as one of the key factors precluding optimal motor recovery[4–6]. In this same vein, a recent connectome-based predictive model also highlighted the role of ipsilesional prefrontal and contralesional cerebellar areas as key drivers for motor recovery [7]. In spite of evidence pointing at a major role of extended systems and structures providing cognitive inputs to frontal motor systems, conventional neurostimulation approaches have insisted on modulating the excitability of directly anatomically impaired or contralateral primary motor and premotor areas with inconsistent outcomes. We here explored the pre-clinical potential of a neuromodulation approach targeting instead large-scale network abnormalities arising from post-stroke MCA diaschetic effects affecting cerebellar and prefrontal structures[8]. To this end, we conducted a parallel-arm randomized double blind clinical trial delivering for 10 consecutive days a 20 minutes session of active anodal tDCS on either the ipsilesional dorsolateral prefrontal cortex (DLPFC), the contralesional anterior cerebellum, or the two former sites combined and had them compared across and to a sham tDCS condition. Before and at several time-points following the tDCS regime, we evaluated their impact on motor outcomes while assessing measures sensitive to the contribution of the modulated targets (i.e., via an impact on visuomotor capacities and clinical functional scores) acquired pre- and post intervention.

METHODS

Study Design

The present study (registered in ClinicalTrials.gov: NCT05329818) was designed as a double-blind, parallel, sham-controlled, randomized pilot clinical trial. The study received ethical approval by the local ethics committee of the *Institut d'investigacio sanitària Pere Virgili* (IISPV, Tarragona, Spain); registration number 077/2021. Participants were included in the study after providing written consent to participate accordance with the Declaration of Helsinki and our ethical approved protocol. The reader is referred to the pre-registered publication for a detailed overview of the study methods[9]. Participants were randomly assigned to one out of the following stimulation 4 groups, GROUP 1: (ipsi-DLPFC) anodal tDCS stimulation of the ipsilesional dorsolateral prefrontal cortex, GROUP 2: (contra-CEREB): anodal stimulation of the contralesional cerebellar cortex; GROUP 3: (ipsi-DLPFC+contra-CEREB) combined anodal stimulation of the ipsilesional DLPFC and the anodal contralesional cerebellar cortex and, GROUP 4: (SHAM tDCS) sham/placebo stimulation. MRI structural (T1-weighted) scans were obtained) in a majority of our patients before the onset of anodal tDCS interventions. For all groups, a daily tDCS session was administered for 10 consecutive days, with a regime of 5 sessions per week for 2 weeks (Monday to Friday), while clinical outcomes were taken at three time-points: I) Baseline assessment (W0), 72h-96h (2-3 days) before tDCS regime onset; II) Post-tDCS assessment(W3), 72h-96h (2-3 days) after the end of the treatment regime; and III) Follow-up assessment (W7), 30 days after the end of the treatment regime.

Participants

The following inclusion criteria were established as to be fulfilled: (1) to have received a diagnosis of middle cerebral artery; (2) to be enrolled in a "live" rehabilitation program in the Rehabilitation and Physical Medicine department of our institution; (3) an age between 18 and 85 years old; (4) to have suffered a stroke within 4 and 12 months prior to enrolment. Exclusion

criteria included unstable medical condition, contraindications to NIBS according safety guidelines and severe cognitive impairment. Participants were enrolled through the Rehabilitation and Physical Medicine department of the *Hospital Universitari Joan XXIII* in Tarragona, Spain.

tDCS Stimulation

HD-tDCS was delivered using an 8-channel transcranial current stimulation and electroencephalography (tCS-EEG) device (Starstim-8®, NEelectrics) providing a maximum current of 4 mA, and controlled wirelessly (via WIFI) with a PC computer with an *ad hoc* control software (NICLAB 2.0, NEelectrics). During stimulation sessions, participants wore a neoprene cap ensuring correct placement for tCS/EEG round electrodes (NG pistim®, πcm2 surface, Ag/AgCl) embedded in SignaGel® in pre-established EEG 10-20 system scalp positions. Anodal tDCS current intensity was linearly ramped up during 30 secs to reach peak current levels associated with each specific condition (GROUP 1: ipsi-DLPFC, 1.736 mA; GROUP 2: contra-CEREB, 1.999 mA intensity; and GROUP 3: ipsi-DLPFC + contra-CEREB, 3.735 mA intensity. Peak current was maintained for a total of 20 min per session. Electrode positions and intensities were based on a previously conducted E-field optimization procedure. For that purpose, a MNI152 based computational model was created in MATLAB and SimNIBS to simulate the electrical field distribution based on participant-specific MRI data. The electrode placements followed the 10/20 EEG system, and isotropic conductivity values were assigned based on previous studies. The solution for optimal stimulation was derived by adjusting electrode positions for the ipsilateral DLPFC and contralateral cerebellum separately before merging them into a combined stimulation setup. For the DLPFC (located at MNI coordinates x=-39, y=34, z=37), the optimal setup used three electrodes, with a maximum current of 4 mA and a safe limit of 2 mA per electrode. The cerebellar stimulation (MNI x=24, y=-66, z=-40) required five electrodes, with the same current limitations. The setup was designed to focus the electric field on the target areas, avoiding regions such as the contralateral temporal cortex and the ipsilateral cerebellum to maintain spatial selectivity. Finally, the optimization procedure incorporated a minimum impact E-field strength at the targeted site, ensuring at minimum 0.25 V/m impact.

Primary and Secondary outcome measures

Lesion features were characterized in every participant. To this end, lesions were manually segmented to then estimate the percentage of damaged tissue within specific brain regions according to the John Hopkins University (JHU) atlas[10]. In order to quantify potential changes in cognitive and motor abilities driven by tDCS stimulation, clinical scales-based evaluation (National institutes of health stroke scale (NIHSS)[11], Fulg-Meyer Assessment (FMA)[12] and the Montreal Cognitive assessment (MoCA)[13]) were administered by trained clinicians at 3-time points (Baseline at W0, post-tDCS at W3 and Follow-up at W7, respectively).

Importantly, we also aimed to identify and quantify the impact of tDCS stimulation on prefrontal (ipsilesional DLPFC) and cerebellar (contralesional anterior cerebellar lobe) systems and functions (sustained-attention and executive control and visuo-motor adaptation, respectively). To that end, a computer-based Visuomotor Adaptation behavioral test was carried out at the same time points as those of clinical scales (W0, W3, W7). Briefly, participants were requested to move a dot from the center of a computer screen to a peripheral target using a hand-joystick operated with their impaired arm. The task included two blocks: a baseline block with 32 trials, and a motor adaptation block with 64 trials during which a 30° angular rotation was introduced to challenge visuo-motor adaptation skills. Crucially, each trial had to be completed within a maximal time window of 10 seconds, adapted to our patient population.

Data analyses and statistical comparisons

The distribution of clinical and cognitive assessment data (FMA, NIHSS and the MOCA scores) was inspected with the Shapiro-Wilk method. Following, statistical comparisons were conducted employing a two-way ANOVA followed by paired Friedman tests (for within-group comparisons) or Kruskal-Wallis (for between-group comparisons). Bonferroni or Dunn's test for multiple comparisons were performed. Visuomotor adaptation trial-by-trial datasets were processed using in-house MATLAB (MathWorks Inc. Natick, USA) scripts. For every trial we estimated the area under the curve (AUC) of the difference between the ideal linear trajectory at

peak tangential velocity (PV) and the trajectory observed (i.e., also referred to as angular trajectory error). Finally, mean AUC values for the last 32 trials (reflecting adaptation towards perturbations) of the task were statistically compared within and between groups.

RESULTS

Twenty-seven participants (n=27) were finally included after medical screening (mean age 61.35 ± 12.98SD (33-78 range); 19 male / 8 woman; 22 ischemic / 6 hemorrhagic, mean months since stroke 6.2 ± 4SD (3-12 range); 14 left hemisphere / 13 right hemisphere) with a first MCA symptomatic stroke according to inclusion criteria. Among such, we recovered exploitable MRI sequences for n=23 of them. Lesion volume varied greatly across patients (Minimum-Maximum range from 0.92 to 204.1 cm3; Mean=62.1 cm3, Standard Deviation= 58.2 cm3) (Figure 1.A shows the overlap of lesion topography across patients; Figure 1.B summarizes the distribution of lesions within anatomical regions). Single participant lesions and the descriptive percentages for each patient are visible in Supplementary Materials 1. No statistical differences were observed between groups during baseline (W0) clinical evaluation (according to primary outcome measure FMA - upper extremity motor assessment) after subject randomization, however note that we dealt with important differences in functional capability across participants, since to facilitate recruitment, that no major restrictions with regards to the severity of the baseline clinical motor impairments were established as part of the inclusion criteria (see clinical scores and within-groups standard deviations of W0 in Table 1).

In order to identify the clinical potential of the multitarget cortical stimulation by comparing the effect of the three active tDCS interventions/groups (anterior cerebellar lobe stimulation, dorsolateral prefrontal stimulation and the combination thereof) driving clinical improvements of upper limb motor function in stroke patients, first order within group and between group clinical results were explored. Behavioral results revealed clinical improvement in all tested clinical domains (NIHSS, MoCA, FMA) evaluated via clinical scales (Table 1).

Statistical comparisons within groups across time-points revealed a significant improvement (Q=10.57, p=0.001) in GROUP 3 (ipsi-DLPFC+contra-CEREB) on general MoCA scores, showing significantly improved (p=0.007) post-tDCS scores (W7) with respect to baseline (W0) performance. Statistical comparisons between tDCS groups were used to disentangle the neuromodulation protocol or protocols leading to higher motor function improvements. Group comparisons revealed on the FMA-LE a potential trend (H=6.5, p=0.08) towards an increase for GROUP 3 (ipsi-DLPFC+contra-CEREB) group, with significantly higher scores (p=0.05) post-tDCS (W3), with respect to baseline (W0) assessments. No other significant effects between groups were observed.

Following, to evaluate the influence of such interventions on specific cognitive and motor processes sensitive to the contribution of the modulated targets (DLPFC and cerebellum), we analyzed the outcomes of the visuomotor adaptation task. A within-group comparisons of the mean group AUC scores encompassing the last 32 trials of the learning curve revealed a significant decrease of deviation trajectory, suggestive of improved motor adaptation abilities ipost-tDCS (W3) compared to baseline (W0) for all groups including sham except for the combined stimulation strategy (Group 1: ipsi-DLPFC, H=5.0 p<0.0001; Group 2: contra-CEREB H=8.0 p<0.0001, Group 3: ipsi-DLPFC+contra-CEREB., H=2.0 p=0.9; Group 4: Sham H=6.4 p<0.0001). Comparisons between groups were employed to disentangle which tDCS protocol led to higher levels of visuo-motor adaptation. This analysis yielded a significant decrease of the magnitude of the trajectory error following cerebellar stimulation (Group 2: contra-CEREB, with significantly higher post-tDCS scores (W3) (H=8.2 p<0.0001) compared to the other anodal tDCS groups (see Figure 2.A and Figure 2.B).

DISCUSSION

We here present an interim analysis of a study embedded in an ongoing clinical trial (E-brain study protocol [9]) assessing the immediate (2-3 days after the last session, W3) and mid-

term effects (2 weeks after treatment offset, W7) of three 10 day regimes of daily tDCS stimulation with regard to their ability to improve motor function according to the Fugl-Meyer Scale.

Recent studies suggest that cerebellar stimulation techniques designed to enhance cerebellar output, such as tDCS and intermittent TMS, promote sensorimotor adaptation and improve gross motor skills in post-stroke individuals [15–25]. Additionally, prefrontal anodal stimulation has emerged as a promising approach to enhance attention and cognitive control in post-stroke patients [26–30]. Despite multifocal stimulation has been shown to increase cortical excitability more effectively than single-site tDCS applications [31–37], no tDCS protocol has been developed yet to simultaneously target multiple brain regions critical for motor recovery modulating globally multiple networks to achieve a state optimal recovery.

Adopting a hodological, network-distributed, view on the causes of motor impairments following brain damage [3,4,14], we designed and tested three novel active anodal tDCS approaches aiming to facilitate motor recovery in patients undergoing in parallel, conventional rehabilitation programs. Notably, our multifocal stimulation strategy was based on targeting the ipsilesional Dorsolateral Prefrontal cortex subtending cognitive control and sustained attention and the contralesional Anterior Cerebellar lobe involved in visuo-motor adaptation. The rationale behind targeting distant areas was two-fold: (1) on the one hand to boost activity in regions relevant for motor function outside of the motor and premotor regions, which were anatomically separated but potentially affected by diaschetic effects and disconnection [8] such as the dorsolateral prefrontal cortex and the anterior cerebellar lobe; (2) to facilitate high-level cognitive processes, such as sustained attention/cognitive control and visuomotor adaptation skills, subtended by these two regions that may both contribute positively to drive motor recovery.

Our clinical outcomes on the basis of n= 27 patients divided in 4 groups (hence n=6-7 participants/condition) suggest a weak trend towards superior improvement in the Fugl-Meyer scores for the upper limb following combined prefronto-cerebellar stimulation. We hypothesize that such effects, if statistically confirmed after the recruitment of a larger cohort of patients,

could be explained by the contribution of dorsolateral prefrontal structures in goal-0directed movements. As observed via within group comparison, combined fronto-cerebellar stimulation (Group 3) yielded higher levels of improvement on cognitive control, according to specific MoCA scores assessing this function. If this outcome is robustly confirmed statistically after adding additional patients to our trial, we here hypothesize that this could be explained by the fact that in normal daily life, optimal goal directed motion is prepared, assisted and facilitated by contributions from the DLPFC, increasing sustained attention, focus and executive control on ongoing voluntary motion hence favoring the conventional rehabilitation process in which patients are engaged. In other words, we here hypothesize that anodal tDCS particularly when delivered simultaneously on ipsilesional prefrontal regions and contralesional anterior lobe cerebellar structures might more likely enable an increase of cortical excitability, which ultimately results in clinical improvements.

In contrast, with regards to visuomotor adaptation outcomes, combined anodal tDCS stimulation of these same targets (Group 3) was the only experimental condition showing significant increases in error variability 2-3 days after the discontinuation of the tDCS treatment (W3) compared to baseline values (W0) (Figure 2.B). Also importantly, anterior cerebellar stimulation (Group 2) was the therapeutic option that reduced the most this same outcome measure assessing visuomotor adaptation processes. Prior research has shown that, for pure sequential learning scenarios, the coordinated timing of cortical activation is critical for motor learning[14,15]. Likewise, during sequential motor learning, spatial coordinates (anticipation and planification of a movement sequence) take precedence during memory consolidation [38]. Two parallel loop circuits operate in learning spatial and motor features during motor sequences. Whereas learning spatial coordinates is supported by a frontoparietal-associative striatum-cerebellar circuit, a M1-sensorimotor striatum-cerebellar network subtends learning motor coordinates. However, visuomotor adaptation skills are considered non-sequential tasks [39,40] in which participants are required to perform the motor tasks and adapt to rotation-like perturbations introduced during the motor execution without planning. In such conditions,

learning is forced by error control and reduction (not by planning) for which a coordinated recruitment of a M1-sensorimotor striatum-cerebellar network, and the decrease of activity throughout frontoparietal-associative striatum-cerebellar circuit are essential. In such context, the variability of increases in spatial deviation observed in our data during the motor trajectory (error increase) under combined anodal tDCS stimulation of the ipsilesional prefrontal cortex and the contralesional anterior cerebellum could be explained by a perturbation of networks involved in motor consolidation caused by transient patterns of non coordinated oscillatory activity across two parallel systems, the above-mentioned frontoparietal-associative striatum-cerebellar circuit and the M1-sensorimotor striatum-cerebellar circuit.

The cerebellum is known to play a central role, anatomically connected with prefrontal, motor and occipital regions, coordinating information integration and transfer [41,42]. Causal cerebellar anodal stimulation with tDCS improves visuomotor adaptation, reducing errors during sensorimotor learning (AUC reduced) [43]. Importantly, prior studies report that faster learning curves can be reached when tDCS is delivered synchronously with movement onset, whereas contrast, tDCS delivered randomly prior to movement onset perturbs adaptation and learning [44]. In that context, the superior learning rate observed in our trial following cerebellar anodal tDCS stimulation o could strongly suggests that the independent stimulation of the M1-sensorimotor striatum-cerebellar circuit is more effective boosting pure non-sequential motor learning in a clinical population.

In summary, we here provide preliminary evidence that combined bifocal anodal tDCS stimulation of the dorsolateral prefrontal cortex and the anterior cerebellar lobe may facilitate motor recovery in stroke patients, and at the same time, perturb visuomotor learning abilities. At this stage of the protocol, the limited number of recruited participants randomized to each tDCS group (n=6-7 patients/group) severely limits the power of our study, making all outcomes fragile and to be taken cautiously. Alternatively, cerebellar tDCS stimulation might be more sensitive boosting visuomotor learning. Additional patients will be needed to increase the power of our study and be able to conclude more solidly on the potential benefit of anodal tDCS stimulation

on prefrontal and/or cerebellar systems improving motor function following stroke. Additionally, future behavioral and neuroimaging studies will be necessary to better characterize anatomically, functionally and neurophysiologically (coding language) communication between these two structures and primary motor and premotor systems, and on such basis design more effective neuromodulation strategies.

COMPETING INTERESTS

The authors declare no competing financial interests.

DATA AVAILABILITY

The datasets produced and/or analyzed during the current study are not publicly accessible due to institutional regulations. However, they can be obtained from the corresponding and/or first author upon reasonable request.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

XC-T, AV-C and MTC conceptualized and designed the trial. MF and RMS recruited the subjects and directed the hospitality organization. XC-T, DS-P, AV-C and MTC, performed the tDCS interventions, collected and analyzed the data. XC-T wrote the first version of the manuscript. All authors read and approved the current manuscript. We thank Dr. Cecile Gallea, Dr. Martina Bracco from the ICM MOV'IT team and Mr. Benoit Beranger, From the CENIR platform for having shared with us the Matlab code of the visuomotor adaptation task and for generously contributing their expertise and tools in the analysis and interpretation of the datasets and Prof. Monica Toba from the Université de Picardie and the ICM Frontlab Team for thorough advice and training in gray and white matter MRI lesion analyses to DS-P.

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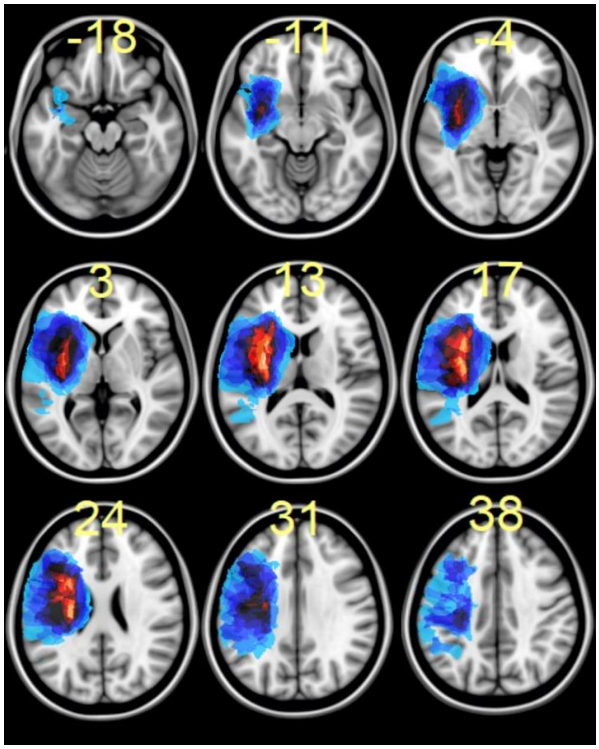
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FIGURES

Figure 1. Lesion anatomy. MRI image lesion topography overlap for 23 subjects. Lesion masks are represented in the left hemisphere for visualization purposes. A. Topographical normalized (MNI152 space) lesion overlapping of 23 middle cerebral artery stroke lesions (19 ischemic / 4 hemorrhagic). Red clusters indicate maximal overlapping voxels across subjects, while blue represents minimal overlap. B. Anatomically based description of maximal overlap across grey and white matter structures. Structural impact was based on John Hopkins University (JHU) atlas. Lesion overlapping graph is organized according to the impacted region (y-axis) organized from top to bottom according to the most commonly impacted structures across subjects, superimposed to the subject lesion extension (x-axis) organized from left to right according to the single-subject lesion embracing more brain regions. Heatmap blue colors correspond to the extent of the lesion within each brain region, hence darker colors represent a lesion covering the totality of the anatomical brain region, while lighter colors represent smaller damage or the absence of damage . Brain regional abbreviations and tables with percentages for each subject can be found in Supplementary Materials 1.

A. Lesion topography overlap



B. Lesion anatomical overlap

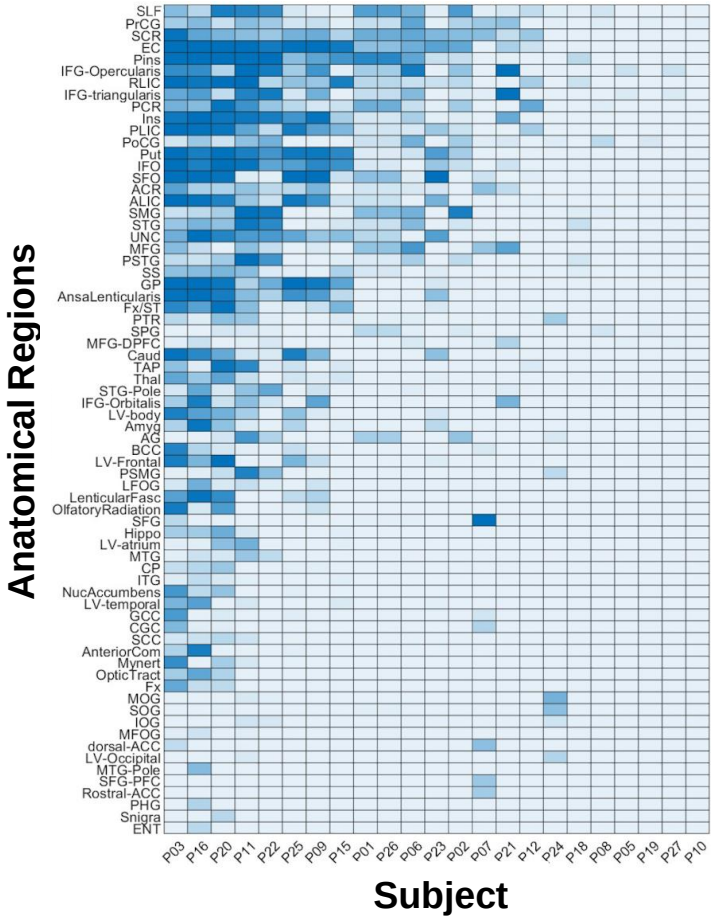
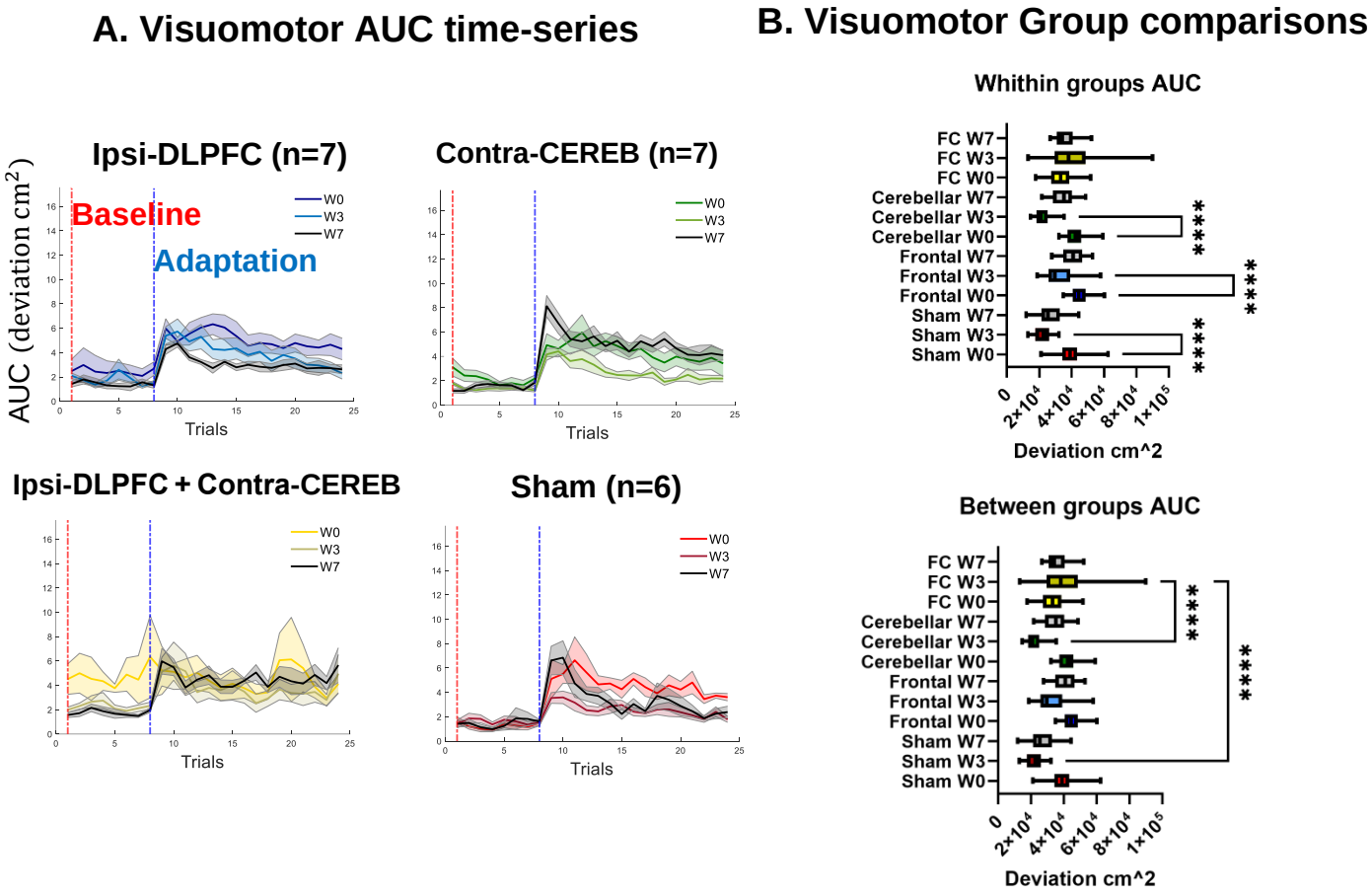


Figure 2. Visuomotor results. A. Visuomotor AUC time-series across groups and evaluation time points. The group mean and standard deviation (±SD) values per each condition and time-point are indicated with a prominent centered line and a subtle background line, respectively. B. Statistical results of the mean group AUC scores of the last 32 trials of the visuomotor task. Evaluation time point at week 0 = W0, week 3 = W3, Week 7 = W7. FC= ipsi-DLPFC+contra-CEREB group, Cerebellar=contra-CEREB group, Frontal= ipsi-DLPFC group. Highly significant comparisons are uniquely displayed for visual illustration. **** p<1x10^-5.



TABLES

Table 1. Clinical results. Group mean (±SD, n=27 / Sham n=6 / Cerebellar n=7 / Frontal N=7 / Fronto-cerebellar n=7) clinical scores for the National Institutes of Health Stroke Scale (NIHSS), Fulg-Meyer Assessment upper extremity (FMA-UE), Fulg-Meyer Assessment lower extremity (FMA-LE) and the Montreal Cognitive assessment (MoCA). Means are reported for every evaluation time-point (pre-tDCS regime W0, post-tDCS regime W7 and 30 days- post-tDCS follow up W7). Within-groups (Friedman tests) and Between-groups statistical comparisons (Kruskal-Wallis tests) for all clinical scales are conveyed.

CLINICAL SCORES					WITHIN GROUPS STATISTICS			BETWEEN GROUPS STATISTICS			
Test	**Stimulation Group**	**Week 0 (W0)**	**Week 3 (W3)**	**Week 7 (W7)**	**Q-Test**	**p-Value**	**Multiple comparisons**	**Comparisons**	**H-test**	**P-value**	**Multiple comparisons**
NIHSS	*SHAM tDCS*	5,43±2,9	3,67±1,3	3,67±2,4	6,952	0,0298*	ns	W3-W0 difference	1,493	0,6839	ns
	ipsi-DLPFC +contra-CEREB	6,17±5,6	5,83±5,7	4,25±4,5	2,952	0,2490	ns	W7-W0 difference	2,382	0,4970	ns
	Contra-CEREB	7,83 ± 4,7	6,83 ± 3,0	7,00 ± 3,3	0,1000	0,9856	ns	W7-W3 difference	0,7676	0,8572	ns
	Ipsi-DLPFC	6,00 ± 7,1	5,00 ± 6,8	4,75 ± 6,4	5,600	0,1111	ns				
MoCA	*SHAM tDCS*	21,00 ± 4,5	24,14 ± 3,7	24,29 ± 4,3	8,667	0,0113*	ns	W3-W0 difference	2,904	0,4067	ns
	ipsi-DLPFC +contra-CEREB	19,67±3,8	21 ± 4,7	22,60 ± 3,7	10,57	0,0010**	W0 vs. W7 [p=0.0073]**	W7-W0 difference	4,153	0,2454	ns
	Contra-CEREB	18,00 ± 4,3	19,33 ± 4,6	19,33 ± 5,9	1,810	0,4599	ns	W7-W3 difference	3,729	0,2923	ns
	Ipsi-DLPFC	18,50 ± 4,5	19,50 ± 6,0	19,00 ± 7,3	0,9333	0,7778	ns				
FMA-UE	*SHAM tDCS*	43,57 ± 18,2	48,5 ± 15,2	44 ± 15,3	4,455	0,1687	ns	W3-W0 difference	2,341	0,5047	ns
	ipsi-DLPFC +contra-CEREB	29,33±21,9	31,17±23,1	27.80±21,8	5,429	0,0628	ns	W7-W0 difference	0,7483	0,8618	ns
	Contra-CEREB	26,17 ± 16,4	28,67 ± 13,6	30,00 ± 12,3	1,727	0,4774	ns	W7-W3 difference	0,7757	0,8553	ns
	Ipsi-DLPFC	41,25 ± 27,5	42,25 ± 27,3	43,75 ± 28,8	0,6667	0,9444	ns				
FMA-LE	*SHAM tDCS*	25,86 ± 7,8	26 ± 7,8	28,5 ± 3,9	1,680	0,4739	ns	W3-W0 difference	6,550	0,0877	ipsi-DLPFC +contra-CEREB group (p=0,05)*
	ipsi-DLPFC +contra-CEREB	23,17 ± 8,0	24,67 ± 9,5	22,80 ± 10,0	2,100	0,4095	ns	W7-W0 difference	1,693	0,6385	ns
	Contra-CEREB	19,00 ± 6,2	21,67 ± 7,3	21,33 ± 9,2	2,696	0,3017	ns	W7-W3 difference	1,344	0,7188	ns
	Ipsi-DLPFC	27,25 ± 9,4	26,25 ± 11,3	27,25 ± 7,3	0,8000	0,7778	ns				

*p<0.05; **p<0.01; ***p<0.001; ns= no significant. Q-test = Friedman Test, H-test = Kruskal-Wallis tests

5. GENERAL DISCUSSION

5.1. Summary of the main results

The human brain works as an activity-dependent system with the ability to remodel and adapt its own structural and functional capabilities to better process and compute external and internal inputs. Such an intrinsic property of the nervous system, dubbed *neural plasticity*, is mediated by a set of biological mechanism enabling resources of the peripheral and central neural resources to overcome limitations of their architecture by reshaping its own original structural and functional organization. The consolidation of novel synaptic interactions, most likely via the '*unmasking'* of previously silent connections or the '*sprouting'* of new collaterals and *de novo* synaptogenesis, and the suppression (via for example synaptic *stripping* mediated by astrocytes projections) of consolidated connections, subtend adaptive changes ensuring the acquisition of novel skills essential for daily life[1,50,51,79]. Nevertheless, these same mechanisms which provide the nervous system of flexibility to overcome their structural and functional *'status quo'* can also be the cause of maladaptive changes in post-stroke neurological disorders. Identifying which mechanisms govern adaptive vs. maladaptive plastic changes, and on such basis define interventional neuromodulation strategies (such brain stimulation) to boost activity leading to adaptive changes while limiting the risk of maladaptive reorganization has become one of the key missions in rehabilitation medicine.

The work presented in this doctoral dissertation addressed within this framework several hot topics in the domain of stroke recovery, plasticity and non-invasive - neuromodulation, with the long term goal to develop the use of neuromodulation strategies suited to manipulate extended neuronal networks through weak electric/magnetic fields. More specifically, the research presented in this thesis investigated: (1) Which are the most plausible strategies to effectively modulate motor brain activity following a stroke with an emphasis on gait and posture, and how to best reveal neurophysiological local neuromodulatory effects of NIBS (Experimental Section 1, Manuscripts 1&2); (2) Which can be the impact of very-weak to weak intensity TMS well below individual resting motor threshold compared to conventional subthreshold intensity patterns modulating local and network motor activity (Experimental Section 2, Manuscripts 3,4&5); last (3) May the use of weak intensity polarisation via anodal tDCS applied to spared single site (DLPC or Cerebellum) or simultaneously (both combined) to a pair cerebral sites in stroke patients drive improvement in motor function (Experimental Section 3, Manuscripts 6&7).

Summary of Experimental Section 1

As an initial step and according to *the long term goal of this dissertation*, the work presented in Experimental Section 1, Manuscript 1 sought to identify effective neuromodulation strategies for post-stroke movement disorders and to address the potential causes of interindividual dose-response variability. A systematic review was conducted to compile studies utilizing tDCS for modulating gait, voluntary motion and core control in post-stroke patients. Statistical analyses revealed a mild to moderate group-level clinical effect of tDCS on gait recovery. Notably, a wide array of cortical targeting strategies was identified, involving stimulation of diverse cortical regions such

as premotor, cerebellar, motor, contralateral, or prefrontal areas. This variability in stimulation approaches facilitated the identification of strategies tailored to specific subpopulations of patients with common lesion features. For instance, retrospective analyses indicated that patients with extensive lesions and severe motor impairments benefited more from strategies aiming at reorganizing cortical resources, specifically through stimulation of distant but functionally connected regions within motor networks. Conversely, patients with smaller lesions and a mild-to moderate impairment showed greater improvement with strategies aiming to remap or baseline normalize neuronal activity, hence stimulating perilesional structures. In summary, our review highlights the potential of neuromodulatory NIBS strategies as valuable adjuncts in neurorehabilitation. It underscored the importance of causal diagnostics of neurological deficits, personalized neuromodulation approaches tailored to stroke-lesion characteristics and brain-state, and the promise of large-scale network-level interventions for patients with extensive deficits where lesional or perilesional tissue may not adequately support functional recovery.

In line with our previous identified findings and according to *specific thesis aim 1* suggesting the need for personalized therapeutics and adapted brain neurophysiological assessments, the work presented in Experimental Section 1, Manuscript 2 aimed at implementing novel tools for causal diagnostics employing TMS-EEG. Concurrent TMS-EEG offers a unique opportunity to causally measure cortical reactivity and time-resolved connectivity with high temporal resolution, with several applications in basic human neurophysiology and for diagnostic or clinical monitoring. However, given the low spatial resolution of EEG and the technical challenges posed by the combination of TMS-EEG, data interpretation remains challenging and largely context or task-dependent, limiting the clinical potential and interpretation of TMS-EEG. In order to further develop tools allowing to extract more precise local signatures of the brain state, we developed a spatial filtering method. By combining information from TMS coil position, electric field models and computational EEG source reconstruction approaches, we developed a framework to source reconstruct and recover signals from the directly stimulated regions while minimizing the contribution of distant sources to the EEG signal. The developed method was experimentally validated, showcasing potential to uncover local evoked neuronal dynamics while limiting the contribution of distal sources in the recorded signal. The approach may have potential to be employed in post-hoc analyses intended to study local perturbational effects or to inform stimulation in brain-state close-loop scenarios.

Summary of Experimental Section 2

Following, guided by our previously noted findings and according to *specific thesis aim 2* suggesting the need for a better comprehension of TMS biological effects, the work presented in Experimental section 2, Manuscripts 3, 4 & 5, investigated the online effects of very weak magnetic fields in the human brain, the online dose-response relationship between TMS intensity and oscillatory elicited responses, and finally the excitatory/inhibitory short-lasting offline effects of a repetitive application of weak magnetic fields.

On such bases, first experiment of experimental section 2 (Manuscript 3) implemented a *online* design where healthy participants underwent a TMS-EEG

experiment where *single pulse* and *rhythmic* (20Hz, 4-pulse bursts) were delivered to the left motor cortex at very weak intensity levels (3%MSO, ~6%rMT, 5-10V/m), and at conventional high intensity levels for comparison purposes (60%MSO, ~80%rMT, 100-150V/m). EEG results indicated that very weak magnetic fields, induced mu-alpha oscillatory activity in a beta-phase-dependent manner on the primary motor cortex. Differences in the oscillatory modulatory effects displayed by the two opposite intensity conditions tested in our study suggested that they operate through different physiological mechanisms; whereas high-intensity stimulation entrains frequency-specific oscillations, very-low-intensity might amplify spontaneous activity of specific neuronal populations responsible for alpha sensorimotor rhythms.

The second experiment in experimental section 2 (Manuscript 4) investigated the input-output relationship between stimulation intensity and elicited oscillatory brain activity. Following a similar *online* design employed in Manuscript 3, rhythmic 20Hz (4-pulse bursts) stimulation was delivered to the left motor cortex at different levels of relative TMS intensity: 10, 30, 50, 70 and 90% rMT. Results indicated that stimulation at intensities >50%rMT of the resting motor threshold (50%rMT) elicited clear local synchronization signatures, similar in nature, but not necessarily in amplitude, to those associated classically with -near (~90%rMT) or -supra (120%rMT) MEP threshold intensities. On the other hand, 30%rMT bursts desynchronized beta oscillations at late latencies (200-400ms). Overall, our results suggest a non-linear relationship between intensity and oscillatory modulation. According to our initial hypothesis, such non-linear effects might indicate that weak magnetic fields may be biologically operating at different physiological mechanisms than high intensity magnetic fields.

The third experiment in experimental section 2 (Manuscript 5) investigated the *offline* short-lasting modulatory effects of repetitive TMS (rTMS) at very weak intensity (10% relative rMT). For comparison purposes, 50%rMT rTMS was delivered in a control subject. In this occasion, a 10HZ repetitive TMS protocol (40-pulse trains, 40 trains total, with 40-second inter-train intervals) was delivered to the left motor cortex. Measures of corticospinal excitability (single pulse MEP, Short-intracortical inhibition and intracortical facilitation) were recorded before and after the repetitive 10Hz protocol. Results indicated that 50%rMT 10Hz rTMS induced excitatory effects comparable in nature to classical near motor threshold rTMS protocols. Oppositely, 10%rMT 10Hz rTMS stimulation led to different results with modest but significant decrease in intracortical facilitation and decrease in short intracortical inhibition. In summary, the evaluation of short-lasting post rTMS effects induced by weak magnetic fields failed to yield a clear, unidirectional excitatory or inhibitory effect. However, significant changes observed, namely, a reduction in both glutamatergic and GABAergic transmission inferred indirectly from TMS paired pulse studies, indicate a potential modulatory influence of this protocol which should be further investigated.

Summary of Experimental Section 3

Finally, according to *specific thesis aim 3,* the work presented in Experimental Section 3, Manuscripts 6 & 7 sought to explore networked opportunities for neuromodulation of post-stroke movement cognitive and motors deficits. Stroke-induced brain injuries often result in direct structural damage, impairing functions associated with

the anatomical affected areas. However, the resulting deficits are not solely attributable to localized tissular damage but also involve widespread diaschisis that disrupts large-scale brain networks. Our initial systematic review revealed that most clinical neuromodulation strategies targeting functional recovery for the improvement of voluntary motion have yet to fully embrace a network-oriented perspective. On that context, the final experimental section explored new network-based stimulation strategies designed to target large-scale networks affected by diaschisis following post-stroke brain damage. To achieve this ambitious goal, we conducted a trial involving real middle cerebral artery (MCA) stroke patients, where they received a 10-days regime of tDCS stimulation alongside a multidimensional assessment of stroke-related deficits. In order to explore the effects of the stimulation, brain regions involved in voluntary motion not directly affected by the ischemic/haemorrhagic tissular damage but affected by the functional diaschisis were identified from previous neurophysiological findings.

Accordingly, MCA stroke participants were allocated in different stimulation groups receiving stimulation the prefrontal cortex, the cerebellum, the combination of both, or placebo stimulation. Importantly, this study not only aimed to investigate the clinical potential of a novel tDCS regimen but also served a more fundamental physiological purpose: characterizing the anatomical and neurophysiological contributions of the targeted brain regions (prefrontal cortex and cerebellum) to the modulation of behaviour, specifically voluntary motion. Our clinical findings showed greater improvements in cognitive control, as evidenced by specific MoCA scores, and suggested a modest trend towards superior improvement in Fugl-Meyer scores (functional motricity) following combined prefronto-cerebellar stimulation. However, the same type of stimulation was also associated with a perturbation (i.e., increased variability) of sensorimotor learning explored through visuomotor adaptation tasks, where a significant decrease of deviation trajectory errors (thus, increased adaptation) was found in all groups except the combined fronto-cerebellar stimulation when comparing baseline W0 vs. post-tDCS W3, with a higher adaptation on the cerebellar stimulated group. Overall, the diaschisis inspired stimulation approaches explored in our trial represent potential new neuromodulation interventions for post-stroke disorders, but must importantly, our research is a proof of concept of networked neuromodulation. However, networked interventions need to be carefully considered, and different neurological symptoms may need different interventions, targeting individual regions of the brain, targeting a single network or multiple networks.

5.2. Neuromodulation in post-stroke voluntary motion: Experimental section 1

The study of the anatomical and functional underpinnings of stroke-induced movement disorders and voluntary movement impairment, alongside the adaptive and maladaptive plasticity that facilitates or limits recovery, has gained significant attention over recent decades[94,95,98,131]. Voluntary motion relies on the ability to plan, execute, and adjust movements in response to ever-changing external conditions. Lesion location or extension can significantly impair various aspects of voluntary motion, including gait control, one of its most natural and essential expressions[250,251]. For example, lesions can affect brain networks involved in executive functions impairing the

planification of gait or the learning and consolidation of novel motor programs, can directly damage anatomical corticospinal or rubrospinal pathways impairing movement execution, or can affect sensory pathways and peripheric central feedback impairing adaptation.

More recently, longitudinal retrospective analyses of lesion-driven impairments have established that gait, and voluntary motion, emerges from intrinsic excitatory-inhibitory balances within the cerebral and spinal central nervous system and dynamic influences with the peripheral nervous system. Each node, as part of a highly distributed cerebral motor network (M1, SMA, premotor cortex, cerebellum, striatum, basial ganglia), contributes to a complex dialog and set of motor sub processes (initiation, maintenance, error control as well as learning, planning, consolidation of human gait, etc.). Dialog across brain regions is modulated and gated through mutually trans-callosal long-range intra-interhemispheric interactions able to maintain homeostatic excitatory/inhibitory balance in areas of the CNS[252–258]. Roughly speaking, after a stroke, the long-range homeostatic balance is disrupted, which can trigger maladaptive plasticity processes. Early acute increases of the perilesional intracortical inhibitory tone may be beneficial for prognosis in post-stroke sensorimotor deficits. However, depending on the system's resilience perilesional inhibition can be beneficial or limiting for mid to long term prognosis. In patients with mild to moderate lesions and functional motor impairments, early perilesional increased inhibition followed by a late downregulation of the former and up-regulation of excitatory agents in perilesional areas facilitates neuronal survival and adaptive plasticity. However, in patients with severe and extensive lesions and levels of motor paralysis with compromised recovery, hence in need of plastic reorganization via distant structures, prolonged long-lasting perilesional inhibition and distant up-regulation of excitatory agents can be beneficial[89,93,97,102,122,126].

Therefore, identifying whether perilesional or long-range interconnected brain areas play an adaptive or maladaptive role for each individual patient has become critical for prognosis and for guiding neurostimulation and rehabilitation strategies. For such purpose, NIBS can be used to assess and monitor underlying levels of excitation and inhibition through paired-pulse or single-pulse protocols, with or without concurrent EEG. Likewise, it can also modulate neuronal activity using repetitive TMS or tDCS protocols (across multiple sessions) to explore whether such modulation (e.g., increasing excitation in perilesional areas) supports adaptive behavioural changes in gait and voluntary motion rehabilitation.

On that context, our systematic review identified 28 studies that employed tDCS with a neuromodulation purpose and demonstrated that gait recovery can be achieved by directly increasing the excitability of perilesional regions in the damaged hemisphere; by suppressing homotopic spared regions of the contralesional hemisphere; the former and the latter simultaneously, or finally by activating spared cortical cerebral or cerebellar regions connected with perilesional areas. However, the effect sizes of the studies included in our review were modest and showed considerable variability. A key source of this variability may stem from the fact that none of the studies incorporated causal testing of the underlying brain state to determine whether the targeted brain areas would benefit more from stimulation strategies aimed at increasing or decreasing activity. As mentioned above, dynamic physiological changes along the recovery process (from subacute to chronic phase) can support or limit adaptive plasticity[93]. Currently, there

is no international agreement on causal testing procedures or on the stratification/individualization of stimulation strategies based on these procedures. In gait recovery, assessing the clinical severity of deficits or exploring the structural integrity of corticospinal pathways (using tractography) can serve a similar purpose. For example, patients with large movement impairments could benefit from excitatory stimulation in cerebellar regions, while patients with low movement impairments could benefit from excitatory protocols in perilesional regions. Thus, incorporating inclusion and exclusion criteria to neuromodulation studies or therapeutical access could be established by baseline clinical outcomes. However, future studies are needed to link the underlying clinical severity, functional state and structural integrity of specific tracts with neuromodulation outcomes to better standardize stratification procedures in neuromodulation.

Our systematic review also revealed that none of the included studies employed procedures for online monitoring of target engagement. Traditionally, neurostimulation for gait recovery in clinical settings has been delivered using standardized protocols, with the same intensity and electrode placement for all participants, same number of sessions and same duration per session. However, to ensure that the intended brain structures are being effectively modulated, online, offline or combined evidence of target engagement is essential. This can be achieved through concurrent neuroimaging (e.g., fMRI), electrophysiology (e.g., EEG), or behavioural tasks that are sensitive to the functions of the stimulated brain regions[144,146–148,259]. Furthermore, previous studies have shown that individualized, prospective planning to optimize stimulation intensity and electrode placement is crucial for enhancing the precision of the targeted region and minimizing unintended effects[228,230,260,261]. Combining neuroimaging and electrophysiological approaches with neurostimulation offers promising opportunities for clinical applications, increasing the ability to predict the effects of stimulation and enabling the adjustment of stimulation parameters in real-time based on physiological responses and clinical outcomes. Finally, our work identified that most neurostimulation strategies aiming at recovering gait and voluntary motion have predominantly focused on modulating corticospinal excitability. Despite this approach has demonstrated clinical potential[173,262], gait and voluntary motion involve not only the execution of movement but also the ability to plan, adjust, correct errors and adapt motor sequences in response to ever-changing external conditions, cognitive skills which are not solely dependent on primary motor areas. In fact, previous research have shown that movement planning and adaptation depend on a broader networks of fronto-parietal and associative cortical regions, where cognitive control plays a crucial role in ensuring proper motor execution[257]. Unfortunately, strokes involving the middle cerebral artery often leads to damage, either through direct structural tissue injury or Wallerian degeneration, of fronto-parietal pathways. However, how cognitive capacities support the recovery of pure motor functions is not completely understood and future studies should investigate the relationship between cognition and motor control.

5.3. Individualized biomarkers for functional neuronal activity: Experimental section 1

Brain stimulation offers new avenues for examining individual anatomical-functional relationships, enabling individualized causal diagnostics and informing therapeutic strategies. The integration and coregistration of neuroimaging correlational techniques (such fMRI, PET, and EEG) with causal methods like TMS or other NIBS modalities, open unprecedented opportunities. However, the integration of these techniques with NIBS remains technically complex, imposing significant limitations on the type and quality of biomarkers that can be extracted.

In particular, the combination of TMS and EEG recordings, even if still far from becoming mainstream in the field of human neuroscience, has gained outstanding popularity thanks to its temporal resolution, and ability for flexible exploration of time-resolved cognitive events in healthy participants and clinical populations[146,150,204,206,263]. However, data interpretation remains challenging and largely context or task-dependent, whereas the accuracy by which physiological markers can be source-localized is controversial. The latter issue is particularly challenging due to interindividual variability in brain topology and the ill-posed nature of the inverse problem (lower number of sensors than independent source generators), and despite its high temporal resolution, EEG is a non-spatially specific mapping method[22,188,264].

To understand the source-origin of evoked electrical potentials, prior computational research has employed dipole fitting approaches, beamformers or minimum norm estimators to determine source origin of post-synaptic potentials captured in EEG recordings[199,202,215,265]. For instance, brain activity can be modelled by focusing on preselected sources presumably generating specific signals of interest, an approach used in dipole fitting. Similarly, beamformers operate under the assumption that only individual sources are active at a given time, using data covariance to estimate the locations of these sources. On the other side, distributed source models, such as minimum norm estimators (MNE) assume that multiple simultaneous sources can be active, but they do not habitually correct for *crosstalk* or *leakage* from nearby or distant sources, limiting precise spatial inference about the true source or sources of brain EEG activity. In the case of concurrent TMS-EEG, stimulation elicits both orthodromic and antidromic signal propagation across interconnected areas, complicating interpretations when source reconstruction methods assume isolated activity in single regions. Additionally, regional propagation can obscure the identification of primary source generators, hence with a risk of false positives and data misinterpretation. Overall, none of these methods are ideally suited for the analysis of time-series from directly targeted/perturbed populations and struggle with the challenge of the multi-source phenomena according to which multiple coactive regions are synchronized, leading to significant *crosstalk* and signal *leakage,* limiting diagnostic and interpretation of TMS-EEG markers.

To improve NIBS tools and more reliably causally explore physiological markers subtending adaptive and maladaptive processes in human brain, this doctoral dissertation aimed to develop, implement and evaluate new technical solutions to accurately estimate EEG correlates evoked/modulated/entrained by TMS, combining computational biophysical E-field modelling with the spatial filtering of EEG signals. Hence, we here developed a computational framework based on cross-talk-functions[203] to supress signal components from outside the boundaries of a predicted

E-field distribution area based on MRI individualized biophysical models of brain distribution. The method presented in Experimental Section 1 (Manuscript 2) was based on previously developed spatial filter approaches for *crosstalk* functions (DeFleCT). Our approach introduced elements to simultaneously preselect sources of interest (pass-bands) or supress sources of noise in specific locations or anywhere in the brain. Particularly in our pipeline, sources of interest are individually selected according to magnetic field distribution. We further validated our method in an experimental setting by targeting the left motor cortex with single pulses. Statistical comparisons were performed between spatially filtered signals vs pre-processed but not spatially filtered signals. Unfiltered signals were extracted from a cluster of electrodes within the stimulated region underneath the TMS coil on the primary motor cortex, according to standard practice. Similarly, unfiltered signals were source reconstructed and recovered from the directly primary targeted region. Such sensor level and source level signals served as “ground truth” for comparison purposes in our analyses.

Non parametric cluster based permutation statistics ($p<0.05$) compared power differences in the time-series (0-400ms) of TMS evoked potentials (TEP), where unfiltered sensor level and source level were compared against the E-field-based filtered signals. Outcomes strongly suggested that the E-field-based spatial filtering procedure, specially when combining with specific auditory contains to minimize auditory evoked activity in the signals, supressed signal components arising from distributed EEG sources outside the predicted area of influence of the stimulation, minimizing the influence of re-entry in M1 of somatosensory and auditory evoked activity sharing also a precentral and central topographic EEG distribution.

With a similar goal to our developed procedures and work, other algorithms have been developed and employed by the TMS-EEG community to spatially filter noise and multisensory contamination from the TMS-EEG datasets. However, to date no consensus with regards to a common standardized pre-processing pipeline for TMS-EEG data in primary motor cortex has emerged[214], and no standardized procedure has been proposed to directly explore local correlates of brain activity emerging from the directly stimulated regions. Procedures employing the SOUND, the SSP-SIR or modified versions of the former adapted to minimize somatosensory peripheral stimulation have been made available for processing TMS-EEG data[209–211] and recover activity from the primary stimulated region and neuronal distant populations. However, results indicate that such algorithms are effective on reducing muscular evoked EEG activity, peripheric activity (somatosensory) and signal noise, but do not address the need of reconstructing signals to study neuronal effects on the primary targeted populations.

Likewise, prior procedures aiming to source-reconstruct local correlates of induced brain activity propose to position dipoles relative to the centre of the TMS coil[266]. However, it remains uncertain whether and to which extent in terms of spatial spread and magnitude, propagation and local/distant synchronization occurs following a TMS pattern. The assumption of absence of event propagation in dipole fitting leads to the underestimating neurophysiological activity, limiting the validity and interpretability of dipole fitting models in certain scenarios. Our E-field-based filtering approach generates a source-specific spatial filter aligned with the E-field's distribution across the grey matter surface. Given the E-field's diffusive nature, with intensity diminishing over distance, this method may better capture local propagative events compared than traditional dipole

fitting techniques. However, future comparative analyses will have to statistically assess differences across reconstructed signals based on several methods, an information that is crucial to understand how different models impact the interpretation of brain activity, enabling a nuanced evaluation of the strengths and limitations of each approach, which can be deemed useful and provide more accurate estimates depending on the experimental design.

5.3.1 Methodological concerns and limitations

TMS-EEG preprocessing pipelines typically involve a series of flexible steps tailored to the specific needs of the experiment and equipment. These steps generally include data epoching, rejection of bad channels, demeaning, TMS pulse interpolation, removal of bad trials, independent component analysis (ICA) to eliminate artifacts from muscle or eye movements, band-pass filtering, re-referencing, and/or baseline correction. Several preprocessing frameworks, such as ARTIST, TESA, and SOUND/SSPR[214], have been developed yielding different results (e.g, final TEPs peak amplitudes) but inferring final comparable data-driven conclusions. Our spatial filtering method can be integrated as an additional step within this preprocessing chain, however it is not exempt of risks and limitations

Our approach assumes that the data has been appropriately cleaned (pre-processed) and originates from a multi-occurring phenomenon (antidromic and orthodromic events propagation) involving multiple coactive sources. This represents, for the first time, a method that enhances the resolution of source localization in such complex scenarios. Therefore, our procedure is more suited in post-processing scenarios aiming to explore temporal neural dynamics of stimulated sources rather than a cleaning pre-processing procedure in itself. Importantly, in our validation experiment, we observed that topographies projecting multisensory activation were supressed. Despite our methods can resemble a cleaning procedure, caution is required when interpreting the results and used with such purpose. Despite the filtering demonstrates an efficient suppression of multisensory artifacts (e.g., auditory fronto-central topographies), the present implementation is designed to directly increase the reliability of local evoked responses, not to suppress multisensory components. Multisensory artifact suppression in our TMS prototypical experiment is a consequence of the seed region (M1) which differs from the multisensory topographical projections (i.e., fronto-central sources). If we had stimulated precentral sources and subsequently applied the spatial filtering, it is likely that multisensory activation would have been amplified in the signal if such activity had not been adequately preprocessed beforehand. When the main objective is to effectively minimize multisensory artifacts in the data, proper experimental procedures employing noise masking, adequate active (or sham) controls[267], and potentially the use of our filters combined with other beamformers and/or cleaning procedures would be necessary[215].

Methodologically, our E-field based spatial filtering grants several opportunities for a more accurate exploration of disease electrophysiological correlates. For example *post-hoc* analyses exploring the contribution of contiguous cortical structures to evoked potentials and/or oscillations (e.g., teasing apart post-central vs. pre-central sources

around the M1 targeted with TMS), long-distance synchronization or propagation of activity by integrating tractography to head models and spatially filtering local and distant sources in the same way with *crosstalk* functions, selecting sources of interest based on the simulation of other NIBS methods such as focused ultrasound or tACS, or, combining the procedure with real time E-field estimation to precisely target local brain rhythms. However, especially important for real-time close-loop stimulation, caution would be advised when applying E-field based filters to signals before the onset of stimulation, since the assumption that the targeted region is solely responsible for the generation of a observed EEG rhythm may not hold always true.

5.4. Functional neuronal effects of very weak magnetic fields in the human brain: Experimental section 2

Efficient medical treatments emerge from a rare combination of a valid pathophysiological model, an adequate characterization of the physiological effect achieved by a medical intervention (e.g., pharmacological drug or an NIBS effects), and a dose-response relationship allowing physicians to predict effects. Regarding the use of NIBS and most particularly, TMS, significant progress is ongoing to understand its mechanisms of action and also defining dose-response relationships when used as interventional tool. However knowledge of the mechanistic principle, integrating several levels of biological organization (from molecule, cell, tissue, organ, to complex systems) remains work in the making.

In particular, TMS effects have been classically explored (and dosed) on the basis of their impact on behavioural correlates and/or two physiological peripheral biomarkers: the *presence, somatotopic localisation, latency, amplitude, intensity threshold, and their modulation thereof*, for objective Motor Evoked Potentials (MEPs) evoked from M1 and also the *presence, intensity, field localisation* and *visual features, intensity threshold,* and their *modulation* thereof of phosphenes elicited in V1-V4 regions[268]. In spite of their wide use, their limitations revealing prove of engagement and inform on some mechanisms of action of neurostimulation has favoured the adoption of concurrent mapping techniques, notably EEG and in a minor extent fMRI to map the acute ("online"), the delayed ("offline") or the longer-term therapeutic effects of NIBS on brain function[147,148].

Nonetheless, the physiological effects of TMS (reviewed in the General Methods section of this dissertation) are not yet fully understood, particularly when human TMS is applied at intensities far beyond (with very weak magnetic fields) than those typically used in conventional TMS studies which index TMS intensity on MEPs or VEPs thresholds, involving high-voltage magnetic fields[13,55,269,270]. Thus, investigating the physiological effects of weak magnetic fields in humans is essential for two key reasons: first, to better understand their neuromodulation potential, and second, to uncover their role in amplifying or interfering with the overall effects of TMS when high-intensity magnetic fields are employed. To this end, this thesis explored the physiological effects of weak magnetic fields (TMS) in the human brain through a series of studies using in all of them individually MRI-neuronavigated low intensity TMS in single pulses, 20 Hz 4 pulse bursts and 10 Hz repetitive TMS patterns, delivered to the left primary motor cortex, (M1) coupled at all times to EEG and EMG recordings.

In a first study, we characterized locally evoked *online* cerebral activity in terms of transcranial evoked potentials (TEPs), Event Related Postsynaptic (ERPS) evoked activity and intertrial phase coherence (ITPC) measures as signs of entrainment and cross-frequency phase dependent effects extracted from concurrent EEG recordings. In an second study we addressed the *input-output* relationship between TMS burst strength from very weak to conventional subthreshold intensities and TEP, ERPS, and ITPC perturbations extracted from evoked the EEG measures. Third and last (3) We explored the longer lasting *offline* neuromodulation effects of a 10 Hz 28 minutes long repetitive TMS protocol via pre-post estimations of cortical and cortico-spinal excitability employing TEPs, ERSP, ITPC and paired pulse measures.

5.4.1 Online effects of weak magnetic fields

The first experiment (Manuscript 3) of Experimental section 2 was designed to explore neurophysiological correlates of TMS-EEG elicited activity when delivered at very weak intensity compared to conventional levels used in TMS human studies. To this end, a cohort of n=18 subjects was included and enrolled in an MRI-neuronavigation-guided TMS session targeting the left primary motor cortex associated to the FDI hand muscle. Participants received high intensity TMS, also referred to as 'conventional' (60% maximal stimulator's output, MSO, ~77% resting motor threshold, rMT), or very weak intensity TMS (3%MSO, ~6% rMT) conditions, and EEG was concurrently recorded in search of evoked or induced responses.

Since the seminal work from Thut et al. (2011)[217] and according to prior research performed in our group and others[37,46,218,271,272], an important EEG feature of TMS phase-locking resulting in oscillatory entrainment is the progressive increase of oscillation amplitude as TMS pulses were accrued across the burst. When the TMS burst does not entrain oscillations but generates instead a repetition of transcranial evoked potentials (TEPs, aka cortico-cortical evoked potentials), a uniform unchanging pattern of amplitudes is present. However, if TMS phase locking builds up entrainment, larger and wider oscillations are progressively evoked. When electromagnetic pulses are strong enough to make individual clusters of neurons fire together, TMS entrains and imposes a frequency specific external rhythm. Oppositely, if TMS power is not enough to make neurons fire, subthreshold fields (w.r.t action potential neuronal thresholds) can still induce modulatory effects, but instead of imposing a frequency-rhythms, weak fields may be operating through stochastic resonance, rhythm resonance, temporal biasing spikes and network entrainment of endogenous rhythms[12,36,37,146,174,175,273].

Accordingly, the outcomes of our first *online* TMS-EEG study showed that high intensity or conventional TMS (60%MSO) revealed classical signatures of local evoked potentials (evoked TEPs) and signs of frequency-specific entrainment with 4-pulse TMS-bursts. Opposite, very weak magnetic fields at 3%MSO intensity did not reveal any sign of power modulation when analysed with conventional metrics such as TEPs, ERSPs or ITPC, as those used for conventional intensity, suggesting no frequency-specific entrainment when TMS is employed at very weak intensity. Nonetheless, previous reports had shown how ups and downs spontaneous fluctuations of neocortical rhythms orchestrate in 'brain state' dependent manner the amplitude of evoked response and the

timing of information flow between inputs and outputs (input-to-output relationship)[18,19,25,30]. On such basis, we hypothesized that very weak TMS intensity could be particularly sensitive to 'brain state' oscillations and accordingly, we decided to explore the influence of oscillatory phase on TMS-elicited brain activity.

To this same regard, it has been suggested that pre-central *mu-alpha* somatosensory (SM) cortex oscillations encodes *input-output* relationship associated to corticospinal excitability, where the trough of such oscillations have been related to local disinhibited (hence more excitable) corticospinal states. Several studies employing TMS-EMG measurements have shown that single TMS pulses aligned with the trough of alpha oscillations increases the amplitude of both MEPs and TEPs elicited by single TMS pulses, suggesting that the alpha trough reflect a sensorimotor state of higher excitability [25,274,275]. However, *mu-alpha* rhythms are thought to have a post-central origin and be originated in S1 cortex, while *mu-beta* frequency rhythm have been related to direct M1 cortical activity[276]. However, the *input-output* relationship between *mu-beta* rhythms and corticospinal excitability is nowadays not clear and remains to be further investigated[44]. Accordingly, we search in our dataset evidence of TMS evoked EEG responses influenced by such rhythms, by sorting signals on a trial-by-trial bases, and comparing the amplitude of elicited transcranial evoked potentials (TEP) and oscillations (ERPS) when either TMS single pulses or 4-pulse rhythmic 20 Hz TMS bursts were aligned with certain phases, notably the peaks or troughs, of the *mu-alpha* or the *mu-beta* rhythms.

Our data reported that 4-pulse rhythmic TMS bursts delivered at high intensity (60%MSO) when aligned to the peaks of the ongoing alpha cycle, resulted in longer lasting beta oscillations. This is at odds with several studies showing that single TMS pulses aligned with the trough of alpha oscillations increases the amplitude of MEP suggesting that the alpha trough reflect a sensorimotor state of higher excitability[25]. Nevertheless, the mechanisms of action of single pulses and rhythmic TMS bursts could differ and be difficult to compare. Hence, a potential explanation for our outcomes could be that 20Hz rhythmic TMS bursts aligned to the peak of alpha oscillations reduces alpha inhibitory bounds, facilitating longer-lasting beta spontaneous reverberation in M1. Such an oscillatory phenomena may occur because TMS can be more effective at imposing entrainment when cortical activity is rather at a silenced state (e.g. at the peak of *mu-alpha* oscillation), a state of a lower resistance by endogenous neural activity which makes such easier to externally impose. In contrast, ongoing cortical rhythms that are excited or disinhibited tend to be more stable and resistant to the influence of new rhythmic inputs with a different frequency[169,174,176]. Delivering stimulation during such active cortical states increases the likelihood of enhancing endogenous entrainment, but may limit the chances of externally imposing a different frequency rhythm.

Oppositely, the same rhythmic 20Hz TMS bursts delivered at weak intensity (hence 3%MSO) aligned to the troughs of the ongoing beta cycle, resulted in shorter lasting alpha oscillations. Importantly, differences on the specific frequency at which such effects were evoked suggest the intracortical presence of mingled mechanisms operating at the level of the oscillators. To this regard, prior research in animal models with weak intensity TMS showed a primary effect on inhibitory interneurons facilitated by their lower resting membrane potential thresholds hence higher sensitivity to increase

their spontaneous activity following perturbations[269]. Moreover, alpha oscillations have been related to increases of inhibitory neuronal activity[34,35]. On such context, we here speculate that our outcomes following weak intensity TMS stimulation could be explained by similar mechanisms hence put forward the notion that neuronal populations with low resting membrane thresholds are particularly sensitive to weak magnetic fields. Thus, weak magnetic fields may induce rhythm resonance and increment the probability of spontaneous release in situations where the 'brain state' (e.g., determined by the phase of a frequency-specific ongoing oscillation) is intrinsically characterized by higher levels of synaptic input into inhibitory populations during the beta phase. In agreement with this hypothesis, when Hi-TMS rhythmic trials were sorted according to the phase of the ongoing beta rhythm, larger and higher TMS-frequency oscillatory entrainment was observed when TMS was aligned to the trough of the beta phase, suggesting trough of the beta phase as a period where brain activity is silenced with less resistance from endogenous oscillatory rhythms. However, further research should investigate with dedicated experiments the underlying mechanistic basis of such phenomena since other explanations could also be plausible. For example, our results could be explained by a more general mechanism, by which local increases of synaptic inputs -hence independently of the predominant oscillatory cycle- refer to higher excitability states inducing a facilitation of the evoked response. Accordingly, peaks of local synaptic input at higher TMS intensity would generate a facilitatory effect on beta TMS oscillations due to the depolarization of pyramidal neurons, while an increase of local synaptic input at lower TMS intensities could generate a facilitatory effects on the alpha oscillations, a process eased by the already mentioned high sensitivity of inhibitory populations (oscillating predominantly at alpha) to perturbations.

5.4.2 Online dose-response relationship of weak magnetic fields

The first study, presented in Experimental Section 2 (Manuscript 3) provided unique insights about potential effects of weak magnetic pulsed fields in the human brain and emphasize their 'brain state-dependency' in terms of oscillation phase. However the specific features characterizing 'dose-response- interactions' between magnetic field strength and the amplitude of transcranial evoked activity (TEPs), evoked oscillatory activity (ERSP) as proxies of entrainment had never been thoroughly investigated.

This is why we designed a second experiment presented in Experimental Section 2 (Manuscript 4), to explicitly map the outcomes of evoked oscillatory activity at increasing levels of TMS intensity from weak TMS intensity to subthreshold TMS intensities used in neuromodulation studies. To this end a small cohort of selected participants (n=4) underwent an MRI based neuronavigation TMS session, in which 4-pulse TMS bursts at 20Hz were delivered to the dominant left primary motor cortex at different TMS intensities all estimated individually relative to the resting motor threshold (rMT). All participants received bursts of stimulation at 10, 30, 50, 70 and 90% of their relative resting motor threshold (rMT), while concurrent EEG was used to record oscillatory evoked/induced responses during the sessions.

Results of this second TMS-EEG *online* study demonstrated that TMS frequency-specific oscillatory power and local synchronization increase linearly according to TMS

intensity. Accordingly, stimulation at 50% of the resting motor threshold (50%rMT) elicited clear local synchronization signatures, associated in nature classically with -near or -supra MEP threshold intensities. Several studies have previously employed TMS stimulation and concurrent EEG recordings to capture transcranial evoked responses (TEPs), at much lower intensities than those conventionally employed to evoke a consistent peripheral MEP response[209,213] (115-120% rMT) or to modulate *offline* M1 intra-cortical and cortico-spinal excitability (80 to 90% rMT)[77]. Similarly, single pulse evoked transcranial evoked potentials (TEPs) are typically found in EEG above 60-70%rMT, indicating cortical evoked activity even at sub-motor resting motor thresholds intensities. Nevertheless, less is known about the repetitive effect when much lower intensities are considered. Based on our results, we argue that intensities at 50% of the resting motor threshold can induce similar physiological effects in nature and direction than conventional ones, however, most probably effects would appear with less power and amplitude and in smaller circumscribed regions.

On the other side, our quest to stimulate at increasing TMS burst intensities, observed that the lowest intensity 20 Hz TMS burst levels -at 10 and 30%rMT- did not show signs of increasing beta oscillations when analysed with classical metrics (TEP, ERSP), but instead 30%rMT stimulation showed signs of beta ITPC beta late latency (200-400ms) desynchronization. Prior TMS-EEG pharmacological studies have shown that late latency beta desynchronization is linked to GABAergic activity increase in the sensorimotor cortex[277,278] suggesting a role for intracortical inhibitory processes as the mechanism of weak and low intensity TMS bursts. Such pharmacological results may align and explain the observed effects of our experiment.

However other potential mechanistic explanations for some of the stimulation levels cannot be totally ruled out at this point. The 30%rMT beta ITPC desynchronization may also indicate that for some trials, oscillations engage in TMS frequency-dependent entrainment (i.e., imposing a frequency rhythm), whereas in some other trials, do not. Underlying factors influencing the probability of a system to get or not get entrained according to the TMS frequency which could fall within the notion of ‘brain state-dependent’ effects have not yet been systematically explored in our analyses. As mentioned, weak electric and magnetic fields influence brain activity primarily through resonance with natural rhythms and by amplifying spontaneous activity, whereas high-intensity stimulation imposes external rhythms onto the system. TMS intensities around 30% of the resting motor threshold (rMT) may operate via both mechanisms, depending on the brain's underlying excitability and ongoing oscillatory activity. However, we were unable to investigate this question in our dataset due to the fixed 20 Hz frequency used in our experiment. Since natural oscillatory rhythms in the motor cortex (M1) typically occur at beta frequencies, distinguishing between imposed rhythms and those that enhance endogenous activity would require TMS frequencies different from the natural beta range. This approach would help clarify whether the observed effects arise from externally imposed rhythms or from the amplification of natural oscillations, as well as the specific brain states conditioning these outcomes (e.g., predominant individual resting alpha frequency, number of conditioning pulses related to oscillatory power, etc.).

Further analyses and eventually additional experiments should carefully explore their bearing on entrainment with an emphasis on the role of stimulation intensity. According to our initial hypothesis, our results suggest a non-linear relationship between intensity

and oscillatory power modulation from the 50%rMT upwards (hence in our case 70 and 80% rMT. Whether the recruitment of entrainment effects via *input-output* curves (plotting TEPs amplitude, EPSPs or ITCPs) across intensity gradients might evolve according to a sigmoidal relationship, similar to MEPs, need to be further investigated.

5.4.3 Offline effects of weak magnetic fields

As important as online effects and 'dose-response' curves tested in Manuscripts 3 and 4, the likeability, magnitude and duration of short- and long-lasting *offline* effects outlasting the duration of the stimulation trains themselves are also paramount to understand the mechanisms subtending weak intensity and use them in specific applications. This is the reason why in a third study (Experimental Section 2, Manuscript 5) we investigated in a small sample of participants (n=4) potential enduring effects of weak magnetic fields on cortical, intracortical and cortico-spinal excitability correlates.

This experiment used a repetitive TMS protocol (10 Hz TMS, 40-pulse trains, 40 trains total, with 40-second inter-train intervals) which delivered at conventional sub-motor threshold intensity in the human M1 cortex (90% rMT) traditionally employed to increase cortico-spinal excitability[77,279–281]. Importantly, our team tested a similar version of this 10 Hz protocol in the barrel cortex (somatosensory whisker's representation) of rodents at very weak-intensity stimulation and showed paradoxically inhibitory *online and* immediately *offline* effects on correlates of neuronal excitability (single cell recordings and local field potentials)[270]. In this context, the aim of our study was to attempt a short-lasting *offline* modulation of cortical M1 activity, and assess such potential effect with metrics of intracortical excitability estimates (paired pulse TMS) and corticospinal excitability measures (MEP amplitude) sensitive to the excitatory or inhibitory effects. TMS intensities were individually in n=3 participants adjusted to 10% of each participant's resting motor threshold (rMT), whereas a 4th subject used as a control received a the same patterns at 50% rMT.

Single pulse pre-post rTMS MEP amplitude changes showed an increase of corticospinal excitability estimates when repetitive 10 Hz TMS was applied at 50% of the individual rMT, hence suggesting that using lower intensities than the conventional TMS intensity (typically 80–100% rMT) has the potential to achieve neuromodulatory facilitation effects comparable in nature (i.e., excitatory), although not necessarily in magnitude and duration to those elicited by conventional (80–100% rMT) subthreshold protocols. Likewise, intracortical facilitation and short-intracortical inhibition paired-pulse measurements indicated a increase of GABAergic facilitation and no modulation of intracortical inhibition, respectively, after the 50% rMT rTMS protocol, suggesting once again comparable excitatory effects in nature than conventional protocols. In contrast, repetitive TMS at 10% rMT led to a modest but significant decrease in intracortical facilitation, as evidenced by lower amplitude paired-pulse MEPs post-stimulation, indicating a reduction in intracortical facilitation. At the same time, we observed a decrease in short intracortical inhibition, as reflected by slightly larger in amplitude MEPs post-stimulation, suggesting a reduction in local inhibition. Despite being statistically significant, the pre-post modulation of MEP amplitude changes in the 10% rMT

stimulated group was marginal, minimal, and *a priori* contradictory, limiting any definitive conclusions about a clear unidirectional effect.

In our previous studies (Manuscripts 3 and 4), we utilized TMS bursts with concurrent EEG recordings at fixed intensities: 3% MSO in Manuscript 3, and 10%, 30%, and 50% MSO in Manuscript 4. In contrast, our offline study (Manuscript 5) employed intensities adjusted relative to the motor threshold (10% and 50% rMT). Motor threshold, assessed in all participants, ranged between 60% and 80% MSO, making 10% and 50% rMT approximately equivalent to 6–8% MSO and 30–40% MSO, respectively. Thus, it is tempting to interpret and draw connections between the offline results from the 10% rMT rTMS condition (in Manuscript 5) and the online results from the 3%-10% MSO conditions reported (in Manuscripts 3 and 4). Similarly, the offline results from the 50% rMT rTMS condition (in Manuscript 5) and the online results from the 30%-50% MSO conditions (in Manuscript 4). However, a direct comparison between the online effects (Manuscripts 3 and 4) and offline effects (Manuscript 5) is challenging in our research. First, the statistical power of these studies differs significantly, with Manuscripts 4 and 5 currently underpowered (n=4) and requiring larger sample sizes. Second, EEG measurements during online TMS capture ongoing oscillatory activity, whereas offline measurements reflect changes in corticospinal excitability. However, the relationship between online evoked oscillatory rhythms and offline excitability modulation effects remains unclear. It is plausible that the former is necessary for the latter, but future research should explore this connection to identify online markers that could predict and guide offline stimulation outcomes. On that context, our experiment suggests a possible relationship wherein, under conditions with online EEG signatures indicating imposed frequency-specific rhythms (e.g., 20Hz bursts entrainment observed in our online experiments at >50%rMT TMS intensities), offline modulatory effects exhibit a unidirectional excitatory pattern for 10Hz rTMS protocols. However, online EEG markers that indicate phase-dependent alpha entrainment at weak TMS intensities do not appear to directly correlate with inherently unidirectional excitatory or inhibitory effects for 10Hz rTMS protocols. Moreover, comparisons between the online and offline effects observed in our experiments are challenging because of the methodological differences and choices. In the online results (Manuscript 3), post-hoc analyses allowed for control of the brain state, specifically the phase of the underlying oscillators. In contrast, the offline study involved delivering rTMS for 28 minutes in predefined patterns, without any control over or information about the brain state during stimulation.

While our primarily focus was to explore differences across rTMS protocols that varied only in intensity, frequency and inter-train intervals, remain crucial parameter for repetitive TMS. Long-time standing evidence has robustly demonstrated that in the primary motor cortex, high frequency stimulation (>=5Hz) delivered at conventional subthreshold intensities indexed to individual MEP thresholds (80 to 100% rMT) induces a lasting excitation whereas low frequency (<=1Hz) results into inhibition[77]. However the frequency-specificity for weaker magnetic pulsed fields as those tested in this dissertation remain quite unexplored.

In a prior attempt, a study deployed alpha (8-13Hz) rhythmic intermittent TMS 25 pulse burst TMS stimulation to the primary motor cortex at weak magnetic intensity[266]. Authors suggested that alpha entrainment in the motor cortex induced local inhibition. However classical intermittent 10Hz stimulation delivered to the human M1 cortex at

conventional subthreshold intensities (~80-100% rMT) have demonstrated solid increases of cortico-spinal and intracortical excitation[77].

Such evidence may align with our results from our offline rTMS experiment presented in Manuscript 5 (with all the required caution given the low number of participants involved), where 10%rMT (n=3) led to a modest but significant decrease in intracortical facilitation, whereas 50%rMT (n=1) 10 Hz rTMS (hence delivered at an alpha frequency) resulted in a lasting local excitatory effect. Therefore, we here suggest that the weak TMS intensities employed rather than the frequency delivered is likely a responsible cause of the inhibitory or suppressive net *offline* effect of 10 Hz rTMS delivered on M1, supporting our original hypothesis of intensity as a critical factor to influence modulatory effects determining net excitatory vs. inhibitory effects.

Moreover, on the basis of such observations, given that very weak (~1-10V/m) or weak (~10-25V/m) subthreshold intensity stimulation may operate by increasing spontaneous firing rates and neuronal entrainment/resonance of endogenous rhythms rather than by imposing frequency-specific activity, frequency may be less relevant for overall modulation effects than in near motor threshold TMS intensities (80-100%rMT). Indeed, mechanisms of action of weak/very-weak intensity TMS might be comparable to transcranial alternating current stimulation, a transcranial electrical stimulation technique operating at low levels of current density (approximately ±0.25 to 0.5 V/m depending on a combination of number of leads, electrode size and set-up intensity)[72,178,282–284]. However, due to the differences in intensity between tACS (±0.25 to 0.5 V/m) and weak TMS (~1-25V/m) at the cortical surface, electrical stimulation is likely more effective at operating through stochastic resonance and temporally biasing spikes to induce phase procession. In contrast, weak TMS may have the capability to not only temporally bias spikes but also facilitate entrainment, depending on the intensity used. This suggests that weak TMS could enhance natural oscillatory frequency peaks, shift these peaks in accordance with the principles of Arnold Tongues, regulate phases to promote more consistent natural oscillatory rhythms, and synchronize cortico-cortical populations. Finally, imposing an external rhythm may only be feasible if the imposed rhythm aligns within the boundaries of the Arnold Tongues[176].

Nevertheless, further experiments crossing variables such as TMS pulse intensity, burst-duration and pulse number and frequency would be needed to better pinpoint the potential entrainment effects. Note that the study 2 (Manuscript 4) and the study 3 (Manuscript 5) of Experimental section 2 present proof-of-concept results from ongoing investigations that could not be completed before the submission of this dissertation. Supplementary data from additional participants and extended analyses including the whole cohort will be necessary to confirm the impact of weak fields on biological brain tissues and further pinpoint the underlying mechanisms driving plasticity. In any case, our studies lay the groundwork for future research and underscore the need to develop a deeper understanding of the mechanisms of action weak and subthreshold magnetic fields in the human brain, which if proven effective modulating brain activity, given their low strength, could be integrated in more autonomous battery-operated multichannel devices with an ability to modulate extended networks and open unique opportunities for the development of new therapeutic applications in neuromodulation.

5.4.4 Methodological concerns and limitations

Several limitations and methodological considerations must be acknowledged in our experimental investigation of weak magnetic fields. First, to study the effects of weak magnetic pulses across all three experiments, we employed TMS-EEG-EMG measurements to capture online cortical oscillations and offline corticospinal excitability modulation. Both online experiments (Manuscripts 3 & 4) have employed intensities under the motor threshold to evoke neuronal activity, captured with concurrent EEG. Oppositely, pre-post excitability measures taken in our third experiment (Manuscript 5) involved supra- motor threshold intensities to generate measurable MEPs. Thus, the measurements extracted from online studies and offline might not represent the same physiological processes and be difficult to compare. For example, during Experiment 1 and 2 employing online designs, stimulation was delivered at subthreshold intensities most likely affecting cortical and cortico-cortical loops generators of underlying oscillatory rhythms. On the other side, Experiment 3 utilized an offline design with supra-threshold MEP measurements, which emerge from a networked effect given that the primary targeted populations by TMS (neuronal populations situated at the crown and lip of the cortex) are indirectly responsible for the MEP generation. In fact, TMS evoked MEPs most likely emerges from the propagation of action potentials across the internal microcircuits of the precentral gyrus, where neurons situated at the wall and fundus of the gyrus, which receive inputs from the superficial crown and lip regions directly stimulated, are secondarily activated and responsible for the downstream propagation generating peripheric MEPs. Hence, Experiments 1 and 2 with concurrent EEG, and Experiment 3 with concurrent EMG, provide insights into distinct physiological processes, and online responses may not necessarily predict offline effects, as the measurements reflect fundamentally different underlying mechanisms.

Moreover, Experiment 3 offline MEP measurements were based on the first dorsal interosseous (FDI) muscle cortical representation, localized with classical motor hotspot procedures. While we employed an MRI-based neuronavigation system able to localize regions and provide steady targeting throughout sessions, we did not incorporated manual or computational network parcellation or the systematic mapping of the muscle representation. In addition to the limitations related to target accuracy, interindividual variability of neuronal thresholds and brain state dependent effects across participants might difficult the interpretations. To address those gaps, computational models of cellular E-field distribution, when paired with experimental validation, could pave the way for a new era of neuroimaging-guided, highly precise neurostimulation protocols able to more precisely correlate biological oscillatory entrained effects with electric-field impact at the neuronal level and TMS intensity. Despite computational cellular models exist and can be nowadays employed to *pos-hoc* simulate single cell responses to external electromagnetic fields in realistic in-silico environments[181], cellular models are still to be biologically validated and theoretical work remains to be corroborated before being systematically employed for prospective TMS planning in human experimental contexts or clinical scenarios.

Our experiments have employed a specific set of TMS parameters (4-pulse bursts, 6-7s Inter-burst interval, motor cortical targets, fixed TMS intensity across the cohort) which can be considered a test bench for neurostimulation, but it could well be that such

parameters may not be optimal to evoked consistent effects across cortical regions within an individual, individuals within a cohort or between different cohorts. For nearly half a century, experimental and clinical approaches in NIBS have tended to set fix stimulation parameters across participants, neglecting, even if well aware, the importance of interindividual differences and the need for parameter customization. It is now clear that such an approach limits sensitivity towards individual effects. Flexibly adapting NIBS parameters, on the bases of anatomical and physiological measures summarized via computational models of current distribution should result into stronger and more consistent effects.

For example, the online study included as Manuscript 3 (Experimental section 2) used 3%MSO intensity for all participants which in turn generated cortical E-fields varying from 3-10V/m when evaluated with biophysical simulations. To the extent that computational models can be considered reliable and validated, individual MRI-based biophysical computational models could be used to estimate the necessary NIBS parameters to generate an equivalent E-field at target in all participants by adapting coil position and stimulation intensity (for weak intensity TMS). Similarly, short rhythmic TMS bursts made of 4 pulses delivered at 20Hz was used for Manuscript 3 and 4 (in Experimental section 2) which was chosen for being the central natural frequency associated to motor systems[44,285]. However as observed in other bands such as alpha or theta from which one has defined the so called individual *alpha (8-11 Hz,* IAF*)* or *theta* frequency (4-7 Hz, ITF) determined at rest or in the context of a specific task, the beta band being much broader (12-30 Hz) than the former might show across-participant variability, therefore individualize procedures to adjust TMS frequency could have been considered. Nonetheless, at difference with alpha or theta bands, the singularisation of single beta peak at rest is far from obvious, it has been hardly used in the TMS or tACS entrainment literature and might have required additional blocks with the practice of a motor paradigm, making our estimations task-dependent and far-removed from the resting conditions in which our studies were carried out, hence not necessarily representative.

Finally, the reader should note that while our studies on weak TMS pulsed fields have focused on local measurements of cortical evoked activity and excitability, the brain is organized in networks with infinite degrees of synchronisation between two extremes (total correlation vs. total anticorrelation). Accordingly, neurostimulation, does not only perturb local activity but also influences network dynamics where the overall effects depend on the state of these interconnected networks[13,90,162,163]. A similar principle applies to brain injury or damage, in which distant structures might be anatomically spared but functionally affected by disconnection or transsynaptic diaschisis, highlighting the broader impact of disruptions in neural connectivity. A vast majority of the NIBS protocols even when targeting a single cortical site are likely to spread to other regions and/or networks, depending on the richness and sign (inhibitory vs. excitatory) projections between regions, the accrued history of prior events they underwent and their activity state[90,162,163]. All our analyses in search of local traces of very weak/weak intensity TMS effects have been focused on direct targeted primary motor systems (M1), traditionally employed as test-bench for exploring the *online* and *offline* neurophysiological effects of NIBS. Hence, at this state of our analyses, and particularly supported the TMS-EEG spatial filter developed in Experimental Section 1 to avoid

sensory and peripheral artifacts, our findings only inform on M1 effects and remain completely 'blind' to more extended network mediated impact.

5.5. Networked neuromodulation in post-stroke motor and cognitive disorders: Experimental section 3

In the third and last Experimental Section of this thesis, we aimed to engage in clinical translation work in the motor domain using weak electrical currents. Previously, in our thorough review (Manuscript 1) of NIB studies in and gait/posture post stroke recovery presented in Experimental Section 1, we sensed that clinical research had opted for tDCS rather than repetitive TMS approaches for neuromodulation, hence trading the flexibility, suprathreshold nature and focality of the latter for the safety profile, low cost and ease of use of the former. Moreover, surprisingly, despite the ability of multichannel tDCS devices to modulate networks targeting multiple cortical sites simultaneously, a large majority of the pre-clinical and clinical trials had opted to target one single cortical region at a time. The combination of weak tCS currents with mild and for many very controversial *online* and *offline* plasticity effects at standard intensities (1.5-2mA), together with suboptimal single site montages, high levels of interindividual lesional variability impinging with the targeted region and limited levels of customization via MRI -based biophysical models of current distribution has resulted in inconsistent and highly variable clinical outcomes. Addressing one of the causes curtailing the efficacy of conventional tDCS protocols, we aimed to explore the combined effects two new stimulation principles to recover upper limb function following a motor stroke: notably (1) To target cerebral structures spared by the lesion even if not directly involved in motor function, but known to subtend cognitive contributions facilitatory of voluntary motion; (2) To supersede the notion of modulating one cerebral system at a the time and seek synergistic effects on motor function via dual site/network modulation. Our strategy aimed to supersede traditional approaches based on the stimulation of a single cortical site/system, either the ipsilesional affected M1 and/or its contralesional counterpart in the spared hemisphere and instead operate in larger scale networks likely affected by *diaschisis* and promote functional reorganization mediated by pre-existing connections.

To achieve this goal, a double blind randomized pre-registered prospective clinical trial, involving subacute to chronic stroke patients with upper limb motor deficits, dubbed E-Brain, assessed three different active anodal tDCS strategies and a sham condition with a multidimensional assessment of stroke-related motor and cognitive deficits tested via clinical scales and computer-based paradigms and EEG recordings. Importantly, the study (see Manuscript 6 and 7 in Experimental Section 3) not only aimed to investigate the clinical potential of a novel tDCS regimen but also intended to causally characterize the anatomical and neurophysiological contributions of the targeted brain regions (notably the ipsilesional dorsolateral prefrontal cortex and the contralesional anterior cerebellar lobe) to the recovery of lost upper limb voluntary motion.

Beyond the modulation of rivalrous inter-hemispheric interactions between primary motor and/or premotor/supplementary motor systems, we predicted that the adoption of a network-based perspective targeting the effects of large-scale disconnections and connectivity-mediated effects via synchronization of structurally spared cognitive networks/systems with bearings on motor function could achieve more efficiently lasting

adaptive plastic changes [15,140,141,286,287]. Said otherwise, we seek to take advantage of the modulatory power of systems/networks structurally spared by the damage and holding connectivity with the affected motor systems as the strategy to modulate the latter and drive motor recovery. Based on the structural and functional reorganization phenomena described in the introduction of this doctoral dissertation, two major strategies had to be crucially considered. The first, one involved the identification of spared attainable cortical targets with intact connectivity linking them to frontal motor execution/control networks. While stroke is responsible for direct enduring tissue damage to specific cortical and subcortical brain regions, local or distant spared areas belonging to the same network receiving white matter (WM) projections from the former can be trans-synaptically disbalanced by *diaschisis*, hence if adequately identified and mapped they could provide an effective target for neuromodulation. More specifically, this strategy requires the extended of viable structural projections and aims to modulate activity in spared cortical regions driving the remapping of function in perilesional viable resources in the stroke *penumbra* region by the *unmasking* of existing connections or alternatively, the generation by *sprouting* of new pathways and synapses [90,92].

The second one (which is complementary and may extend the former), it is based on the notion that the spontaneous or rehabilitation-induced recovery of a motor deficit induced by a focal stroke could be facilitated by preserving and then boosting the neural substrates of high level cognitive functions such as sustained attention, motivation and an ability for motor planning on one side or motor (re)learning and visuo-motor (re) adaptation skills. To this regard, it has been shown for example that clusters of neurological symptoms organised in syndromes following a stroke may manifest or not clinically depending on the location and regional extent of the damage and the impact on crossing pathways connecting distant networks. More specifically, a patient suffering a middle cerebral artery stroke impinging in the WM underlying the right primary motor cortex may present with left hemiparesia due either to tract damage in corticospinal systems, or caused by attentional orienting and awareness deficits secondary to the compression of fronto-parietal WM tracts in the right hemisphere by oedema. As in the subacute and chronic stroke stage oedema resolves and the pressure on this right fronto-parietal WM bundle is released attentional orienting impairment could disappear but left hemiplegia will persist due to the irreversible damage exerted on the corticospinal tract [114,116,117]. If instead the above-mentioned stroke deficits reflect a dysfunctional behavioural output caused by an abnormal state of desynchronization between right fronto-parietal attentional systems and right frontal motor networks, restoring by reorganization the communication between involved networks would drive recovery over time [114–117,288,289]. Indeed, such desynchronization between networks might be a consequence of a large-scale diaschisis effect, impairing normal homeostatic balance and information exchange across motor and frontoparietal networks directly impacted in MCA strokes. According to prior studies, this pathophysiological model is prominent in middle cerebral artery strokes, in which, the preservation and recovery post stroke of attentional orienting skills (spatial and sustained attention; highly involving dLPFC activation) have been found predictive of motor recovery [91,114,116,290–292]. Indeed, pure motor function (movement) does not directly employ fronto-parietal resources associated to attentional orienting in space, however, movement requires precise control and planning of actions in time and space, for which spatial and sustained attentional capacities processed by higher-order cognitive networks are important [130]. Moreover,

during motor planification and execution, anticorrelated activity between fronto-striatal-cerebellar pathways (with a increase of network engagement during planification and decrease during execution) and M1-striatal-cerebellar (with a increase of network engagement during execution and decrease during planification) has been shown to be necessary for successful motor output [257,258]. On such basis, the partial or complete restoration of communication between motor execution/control and attentional spatial orienting or sustained attentional networks can be instrumental for the planning of voluntary motor sequences, contributing to the recovery process after stroke.

In the E-Brain clinical trial we aimed to assess in motor stroke patients the causal impact on motor recovery of single vs. dual-site excitatory modulation to enhance spontaneous activity in motor execution and control systems. To this end, we targeted with an anodal tDCS regime regions not directly damaged by the stroke but sustaining spared WM connectivity with affected motor regions and processing cognitive functions known to be facilitatory for voluntary upper limb motion such as the anterior cerebellar lobe (CEREB) involved in visuomotor (re) adaptation and (re) learning and the dorsolateral prefrontal cortex (DLPFC), playing a major role in sustained attention, reward, motivation and planning. A cohort of chronic motor stroke patients between 18 and 85 years, having suffered a unilateral stroke 4 to 12 months involving the middle cerebral artery prior to enrolment, and participating in an active rehabilitation program, were randomly assigned to one of the following four stimulation groups: prefrontal, cerebellar, prefrontal-cerebellar and sham stimulation. Using a E-field model-based tDCS montage delivered daily during 10 days (from Monday to Friday for two weeks), we assessed at differently stages prior and after the end of the stimulation regime, changes in upper limb motor function with the Fugl-Meyer Score, defined as the primary outcome measure. Importantly, as secondary measures, we used computer based tasks, to assess cognitive changes in the dorsolateral prefrontal function via the AX-CPT such as sustained attention and cerebellar function via a visuomotor (re) adaptation task.

Despite the apparent novelty of the bifocal stimulation approach employed in our clinical neuromodulation study in stroke patients, there is a prior history for this kind of interventions in analogous fields [293]. Similar to our own intervention presented in this dissertation, tDCS strategies targeting either the cerebellum or the prefrontal cortex have been largely considered. However clinical efficacy varied greatly across individuals, likely because of the non-standardization of tDCS methods applied [12,75]. In our trial, strictly following inclusion criteria, twenty-seven subjects (n=27) having suffered a first stroke were prospectively included in the study. Preliminary analyses of intervention specific post vs pre stimulation changes suggests a modest non-significant trend toward superior in Fugl-Meyer scores after a combined prefronto-cerebellar stimulation, which is not found for the other stimulation conditions. Moreover, the group of patients randomized to a combined fronto-cerebellar stimulation treatment also showed post-pre performance increase in cognitive control according to the specific MoCA subscores. If these effects were to be statistically confirmed in a larger cohort and with the use of more restrictive inferential statistical approach, they could causally emphasize the known role of prefrontal structures, and notably the DLPFC in goal-directed movements and/or sustained attention, essential to support rehabilitation process. Indeed, goal directed action involving daily life events requires adaptive planification, exquisite motor control and optimized execution. In such context, the improvements observed in our patients, particularly following the anodal DLPFC tDCS and dual site stimulation condition may

arise from increased prefrontal cognitive control substantiated by higher outputs in MoCA subscores associated to executive functions. Most importantly, they would support prior evidence putting forward cognitive control as an important predictor of motor recovery[91,94,114,116,130,294,295]. However, even if such a hypothesis is very tempting, the neurophysiological mechanisms subtending plasticity processes compatible with clinical outcomes of this kind results remains yet to be demonstrated. Importantly, the combination of neuroimaging (MRI) and electrophysiological (EEG) datasets and clinical performance measures recorded in our study will be in a position once completed to demonstrate signs of network reorganization and neural plasticity rightly associated to behavioural change.

In the last study of this dissertation, we also employed a visuomotor adaptation task to explore changes in motor adaptation and learning abilities -a process subtended by the anterior cerebellar lobe- by anodal stimulation. Excepting for combined prefrontal and cerebellar tDCS, our data revealed significant decreases in deviation trajectory errors suggesting improvements in motor adaptation for three anodal tDCS experimental groups immediately following treatment (W3 vs baseline). Opposite, a regime of combined prefronto-cerebellar stimulation induced significant increases in trajectory error variability comparing these same time points. In our stroke trial, motor function and recovery was properly characterized with well standardized clinical scales such as the upper limb Fugl-Meyer score. In contrast for the assessment of pre-frontally mediated cognitive control or for motor learning contributed by the anterior cerebellum, we had to relie on experimental paradigms such as sub scores of the MOCA and the AX-CPT on the one side and custom programmed visuo-motor adaptation task, respectively [173].

On that context, previous studies have emphasized the crucial role of coordinated cortical activation timing in sequential and visuomotor learning[257]. In sequential learning motor tasks, spatial coordinates—such as the anticipation and spatial planning of movement sequences—are prioritized before movement execution during memory consolidation. Two anticorrelated separate circuits facilitate the learning of motor execution or spatial coordinates: a frontoparietal-associative striatum-cerebellar circuit for spatial learning (increasing activity during planification phase), and an M1-sensorimotor striatum-cerebellar circuit for motor learning (increasing activity during motor execution phase). In contrast, visuomotor adaptation tasks are non-sequential and learning is primarily driven by error correction rather than planning. In these tasks, no specific time window is available for planification during execution, forcing the motor movement to be unplanned. This form of learning depends on reducing errors through active adaptation, which requires enhanced neuronal activation in the M1-sensorimotor striatum-cerebellar circuit and decreased activity in the frontoparietal-associative striatum-cerebellar circuit[251]. Our preliminary findings, particularly if proven robust with a more complete cohort may suggest that combined prefrontal-cerebellar anodal tDCS stimulation condition leads to competition for limited energy resources and a loss of coordination between both networks impairing sensorimotor adaptation. Conversely, anterior cerebellar lobe anodal stimulation delivered in isolation might engage activity in critical M1-sensorimotor striatum-cerebellar connectivity facilitating visuomotor learning [72,73,249,296–300]. Unfortunately, hemiplegia makes the execution of sequential tasks challenging in upper limb hemiplegia patients. Hence, weather dual site anodal tDCS stimulation of prefrontal and anterior cerebellar resources in this same stroke population may have increased performance in sequential learning paradigms remains untested.

A network approach to rehabilitate function in the central nervous system remains challenging but sets the stage for novel exciting opportunities for non invasive neuromodulation in post-stroke rehabilitation therapy. Nonetheless, developing such a new generation of precision neurotherapeutics based on such principles and tools will demand a more accurate characterization of the structural and functional connectivity patterns conveying local and distant synchronization signals across neurons (within and across cortical columns and structures) subtending the processing and exchange of information. The prefrontal and cerebellar bifocal anodal tDCS stimulation approach evaluated for the first time in our trial is a first step towards such a goal and a proof of concept rarely tested before in interventions aiming to restore function in post-stroke motor disorders.

Our trial involved the stimulation of the prefrontal cortex and the cerebellum, administered either separately or in combination. The physiological effects of tDCS have been extensively studied in cortical structures, and much of the knowledge about its mechanisms of action[169] and neuronal spatial sensitivity to external currents[181] can be inferred to our prefrontal target. Notably, anodal stimulation is generally associated with increased local excitability, while cathodal stimulation tends to decrease it in cortical regions. However, the unique convoluted cytoarchitecture of the cerebellum challenges the direct application of these classic tDCS mechanisms to cerebellar stimulation. For instance, studies on anodal tDCS in the cerebellum have yielded opposite results, demonstrating modulation of cerebellar-brain inhibition (CBI) in both directions[301–303]. These inconsistencies can be attributed to various methodological choices combined with the complex anatomical organization of the cerebellum. However, today there is no consensus on the effects of anodal tDCS in the cerebellum. This uncertainty is particularly relevant to our trial, where anodal cerebellar stimulation was applied to stroke patients with the aim of increasing cerebellar activity, which is often diminished by diaschisis effects. While anodal tDCS has shown behavioural improvements in visuomotor adaptation[72, 73, 249, 295], result consistent with our trial outcomes, the post-tDCS physiological effects on the cerebellum remain debated, unpredictable and not possible to infer from our behavioural and clinical results. Cerebellar tDCS effects might be highly dependent on whether the tDCS field primarily influences the cerebellar cortex (with large GABAergic projections towards the dentate nucleus), the dentate nucleus (with rich Glutamatergic projections towards thalamo-cortical loops), or both. Hence, the impact of tDCS on the cerebellum can lead to either inhibitory or excitatory outcomes (e.g., bidirectional CBI modulation), resulting sometimes in unpredictable effects on motor recovery. Future research should prioritize characterizing these phenomena and establishing standardized protocols for cerebellar tDCS modulation. This would pave the way for more effective and reliable clinical applications, enhancing the predictability and therapeutic potential of tDCS in post-stroke motor recovery.

On the other hand, given the clinical nature of our trial, participants received stimulation at rest for 20 consecutive minutes during each session. While this approach is common practice in clinical neuromodulation today, alternative opportunities still to be explored in humans. Several TMS (i.e., supra-threshold) studies have demonstrated that both the immediate and long-term effects of stimulation are highly dependent on the underlying brain state at the time of stimulation delivery[25,274,275]. However, this state-dependency is less clear for transcranial direct current stimulation, as its mechanisms of action rooted in stochastic resonance and the enhancement of spontaneous activity, may

be less dependent of rapid (milliseconds) shifts of cortical excitability. Nevertheless, the ongoing levels of synaptic input, may increase or decrease the capacity of the subthreshold external electrical fields to modulate and facilitate spontaneous activity[12,183,259,304]. Therefore, electrical stimulation should not be confined to conventional 20-minute protocols designed to induce excitatory effects. It remains to be further experimentally investigated whether tDCS can be synchronized with ongoing brain rhythms and behavioral tasks, delivering brief, targeted pulses to functionally activate neural circuits in a precise spatiotemporal manner aligned with task demands.

Finally, note that the complexity and precision of stimulation protocols can be thoroughly explored in experimental non clinical or clinical venues, whereas protocolized easy-to-implement interventions are necessary to test stimulation effects in clinical contexts. Moreover, network-based interventions have to be carefully considered, designed and planned on the basis of a maximum of information on neuroanatomical, neurophysiological and clinical status, keeping in mind that different neurological symptoms require interventions, targeting specific regions or networks. Accordingly, single session pre-therapeutic *proof-of-concept* cross-over studies assessing different conditions in the same population of patients should anticipate ulterior parallel arms therapeutic clinical trials with regimes of periodical NIBS sessions. Last but not least, Indeed, even if not manipulated in the present dissertation, experimental paradigms can be envisioned as a strategy to foster adaptive patterns of neuronal activity priming the brain for more optimal NIBS interventions enhancing both target engagement and network-specific effects. Paradigms of such kind might prove particularly promising in post-stroke clinical research, with immersive 3D environments paired with EEG monitoring may be used to drive brain activity in a way that optimally facilitates the strengthening and/or consolidation of new adaptive and therapeutically meaningful synaptic pathways, in the context of a tailored approach for neurorehabilitation.

5.5.1 Methodological concerns and limitations

The preliminary outcomes of our multifocal tDCS stroke trial steers existing interest in network-based brain stimulation. However it does suffer from limitations that deserve to be thoroughly discussed in this dissertation.

In this study, we included participants with middle cerebral artery stroke in the sub-acute stage (4–12 months post-stroke). Despite this shared timeline, the residual functional motor capacities varied considerably across individuals. At the group level, these differences may have canceled each other out, resulting in no statistically significant differences in baseline clinical performance. However, at the individual level, these variations can have profound implications. As discussed in the introduction, individuals with severe clinical impairments may benefit most from strategies aimed at reorganizing cortical activity to engage intact but distant and previously silent interconnections. In contrast, those with milder impairments may respond better to approaches focused on restoring baseline neuronal activity within the lesioned or perilesional areas. To assess the functional capacity of specific brain regions in driving adaptive or maladaptive behavioral changes, transcranial magnetic stimulation (TMS) protocols, such as intermittent theta burst stimulation (iTBS) and continuous theta burst

stimulation (cTBS), can be utilized. The outcomes of these protocols provide valuable insights into optimal stimulation strategies [97,128,129] offering an opportunity to stratify participants and tailor neuromodulation interventions to their specific needs. In our trial, stratification was not feasible, as the primary objective was to evaluate the effects and contributions of a neuromodulation protocol targeting the cerebellum and prefrontal regions on motor deficits and learning. Consequently, we were unable to identify the optimal neuromodulation strategy, whether perilesional or distant stimulation for each individual, given the study's design. This limitation reduced our ability to ensure that the intervention provided was the most effective in promoting adaptive clinical changes across the cohort. Future research should integrate stratification protocols with lesion severity as a one of the main factors, along gender, age and time post stroke, including neuromodulation strategies better tailored to each individual profile by taking into account all of the above factors and also skull and brain morphology, lesion localisation and its volume. However, in the meantime, prior effort to explore and eventually establish reliable outcomes for the here tested active neuromodulation strategies remains an essential effort, and for this reason it was prioritized as the *core* objective of the present clinical trial.

Another notable limitation of our study lied in the variability of stimulation sessions and the dose received by participants. We implemented a standardized protocol comprising one 20-minute session per day, five days a week, over two weeks with predefined levels of intensity according to computational models. While this approach ensured consistency and robustness across participants in our clinical research, it did not accommodate individual differences in dose-response relationships to stimulation. Moreover, no closed-loop methods were employed to ensure real-time target engagement, given the broader clinical focus of the study. As a result, the total dose received by individual participants may have introduced variability, as it remains largely unknown. This uncertainty likely contributed to greater variability in the outcomes, complicating both inference and cross-group comparisons. Future neuromodulation research should explore the possibility to incorporate online tDCS markers predictive of offline responses and develop adaptive strategies to dynamically monitor and control target engagement during stimulation, adapting tDCS parameters such intensity or session length. Such advancements could enhance the precision and efficacy of neuromodulation interventions. Additionally, while the stimulation regimen was clearly defined, some participants were unable to complete all 10 sessions due to medical or personal reasons. To address this, participants completing fewer than eight sessions were excluded from the final analyses. Nevertheless, variability in the total number of sessions completed may have introduced inconsistencies at the group level, potentially complicating the interpretation and comparability of results. Future studies should prioritize strategies to minimize dose delivery variability and explore individualized protocols to optimize therapeutic outcomes. Tailored approaches that align with participants' unique clinical profiles and capacities could mitigate these challenges and further refine the efficacy of neuromodulation therapies.

Finally, in our study, we utilized a fixed electrode montage for electrical stimulation, based on an MNI152 computational optimization. While this approach relies on a generalized "average" model of human brain organization for targeting precision, individual anatomical differences among participants likely resulted in significant variations in the E-field distribution and impact of tDCS. As previously noted, achieving

optimal stimulation through prospective planning and adjustments to parameters such as intensity and electrode positioning necessitates MRI acquisition and computational modelling prior to stimulation. This limitation was a notable constraint in our study, as the clinically robust design precluded individualized modelling and stimulation. Consequently, the precision of the stimulation may have been suboptimal in some participants limiting target engagement, and in worst case scenarios, modulating activity of concomitant occipital brain regions with different anatomical-functional organization.

5.6. Further considerations

According to the long term objective on which this dissertation is formalized, develop the use of weak to conventional intensity E-fields to modulate networked neural processing driven by excitability modes or oscillations in health and disease, the ongoing research within this thesis proposes new analytical methods to measure effect of NIBS on bioelectrical correlates of brain function and plasticity effects (Experimental Section 1); to deepen our understanding of the physiological mechanisms and principles by which intensity-dependent NIBS, notably TMS, operates (Experimental Section 2); and to explore novel neuromodulation approaches for clinical NIBS applications (Experimental Section 3).

By combining neurophysiological recordings in non-clinical and clinical settings with technical innovation in coupled NIBS and EEG analyses and contributing to novel approaches involving future personalized real-time brain-state-informed noninvasive stimulation and neuromodulation, our work intends to contribute to the emergence of new stimulation technologies and know-how. In the context clinical neuromodulation in stroke patients, it is well known that between-subject variability in brain-state combined with within-subject dynamic changes of the same phenomenon affects the precision and magnitude of target engagement by conventional (TMS and tCS and its derivates tDCS, TACS, tRNS) and emerging (Transcranial Focal Ultrasound Stimulation, tFUS and transcranial Time Interference Stimulation, tTIS) NIBS technologies. Despite no international consensus is established yet, stimulation must attempt to account the best it can for interindividual variability and accordingly, future strategies and routes to evaluate brain excitability and deliver stimulation in specific behavioural contexts will have to be implemented. Such strategies need to seriously consider among other factors lesion localization and extent or volume, the counteracting effect of the neural reserve and resilience to damage and time post-stroke onset. Indeed, as mentioned in the introduction of this dissertation with regards to the latter factor, molecular cascades engaged in perilesional inhibition promote cell survival at early acute post-stroke stages. Thus, according to the outcomes of the current dissertation, during the acute phase of a stroke, inhibitory peri-lesional tDCS, cTBS, low frequency rTMS or low intensity rhythmic TMS and repetitive TMS (at 20 and 10 Hz respectively) could play a potential therapeutic role.

Additionally, along the recovery process (sub acute to chronic phase), increased glutamatergic and reduced GABAergic release have been found responsible for promoting neurogenesis and synaptogenesis [93]. Thus, excitatory perilesional stimulation or to distant structures via high-frequency TMS, iTBS or excitatory tDCS can promote functional reorganization. Additionally, contextually-induced brain-state-

dependent stimulation might boost therapeutic effectivity and open novel opportunities in neuropsychiatry and neurorehabilitation. Currently, the most popular brain stimulation approaches used clinically are transcranial Current/Electrical Stimulation (tCS or tES) and Transcranial Magnetic Stimulation (TMS), which leverage electromagnetic induction and subliminal and supraliminar and cellular polarization to modulate neuronal excitability via an effect on resting membrane potential. Conventional non-invasive brain stimulation (NIBS) such as TMS and tCS approaches used in this dissertation have now well-established uses as tools to causally characterize cognitive anatomy and in combination with fMRI or EEG explore their subtending neurophysiological mechanisms. The role of TMS combined with via EMG, EEG and behavioral biomarkers as diagnostic, prognostic or follow-up, tool for neurology and psychiatry is progressively getting momentum. Last but not least, TMS and tCS keep holding promise for a long list of therapeutic applications by operating at local and network distributed level. In these three facets, combination with a low cost, portable, scalable and temporally accurate tool to monitor *online* or *offline* their effects on brain activity, such as for example EEG, is becoming paramount for any NIBS intervention.

Unfortunately, artifactual interferences between electrically based NIBS and EEG recordings makes the exploitation of such datasets complex, posing *a first major challenge* for the field of neuromodulation, this dissertation has attempted to contribute to (Experimental section 1). Accordingly, the *first experimental section* of this dissertation presented a novel EEG data pre-processing and optimized analytical method which based on the use of E-field model guided spatial filters for such data could also be applied to improve stroke therapeutics with NIBS. To this regard, the analytical method we developed opens the opportunity to explore more accurately with concurrent TMS-EEG approaches the spectral correlates of conditions such as stroke, hence to disentangle the spatio-temporal frequency dynamics subtending pathological performance and its recovery. Emphasis should be also placed on the specific interregional spectral EEG communication patterns associated to motor function, which at difference with motor spatial correlates, are yet to be fully characterized causally. Overall, while technical developments will gradually shape the neuroscientific questions that can be accurately addressed at a given time, it is essential that advancements keep being driven also by core neuroscientific and cognitive inquiries, then translated to the needs of clinical practice.

Conversely, the use of weak magnetic fields for neuromodulation is less conventional, and knowledge about their effects on humans remains limited posing a second *second major challenge* in the neuromodulation field to which this dissertation attempted to contributed (Experimental section 2) exploring the efficacy and specificity of different types of magnetic/electrical fields which could ultimate allow stimulation strategies suited for the modulation of extended neural networks. Despite some of our results should be confirmed and replicated, our findings indicate that neuromodulation approaches specifically engaging inhibitory compared to excitatory populations organised in local frequency-specific oscillators or wider networks might be made feasible in a near future. Stroke therapeutics could integrate strategies to boost local inhibitory systems, combine a modulation of local and distant inhibition, or to spatiotemporally adjust patterns of non-invasive stimulation to excite or inhibit multiple regions according to specific behavioural contexts. To that end, future research will have to delve deeper on the fundamental physiological mechanisms subtending the properties

of circuits and individual neuron type parameters, such as threshold, natural frequency to which contributes locally and phase. Equally important, we must establish the optimal non-invasive stimulation parameters to tune the activity of these neurons types and achieve, cell-type-specific neuromodulation [305,306] in humans. While in rodent models this type of inquiries is becoming mainstream in genetically modified neurons with optogenetic approaches achieving this level of specificity in humans remains out of reasonable reach.

TMS employed at very low intensities offers promising avenues to investigate neuromodulation effects and unravel dose-response dynamics. Despite the differences between tCS and TMS, both methods hold significant potential for clinical applications and for advancing our understanding of the physiological mechanisms underpinning plasticity and the recovery of lost functions. Moreover, TMS weak intensities can be precisely calibrated to achieve specific V/m impacts on cortical surfaces, enabling targeted modulation of distinct sub-physiological process engaged in sub-threshold neuromodulation; allowing for the direct and isolated temporal biasing of neuronal spikes, selective modulation of oscillatory phase shifts, or the enhancement and entrainment of local endogenous oscillations, facilitating the study of their relationship with plasticity and behaviour. This feature represents a significant advantage over tCS methods (e.g., tDCS or tACS), as electrical stimulation primarily operates through stochastic resonance mechanisms due to its relatively lower impact on the human cortex, typically peaking at 0.5–1 V/m. Likewise, weak electric and magnetic fields can be used for diagnostic purposes to assess brain state along the recovery post-stroke period, but therapeutically to manipulate local excitability. In the first experimental section of this thesis, we developed novel neuroimaging methods, based on spatial filters, that can significantly improve stroke diagnostics and inform therapeutics. For example, our method to spatially filter local signatures of brain excitability opens the opportunity to explore causal spectral networked correlates of disease, disentangling precise spatio-temporal frequency dynamics subtending behavioural and clinical performance. While spatial correlates of motor function and learning have been investigated in previous studies, the specific patters of spectral inter-regional communication have rarely been characterized.

The *third major challenge* in the field of causal neuroscience to which this dissertation intended to contribute to (Experimental section 3) is to understand, control, and guide the activity of distributed brain networks to then translate such principles to boost clinical recovery following brain damage. It is precisely within this context, *the third experimental section* of this dissertation examined the effects of dual site tDCS stimulation of prefrontal and cerebellar structures and their associated networks, promoting motor recovery. Although mapping of cognitive processes or neurological symptoms on human brain circuits remains challenging, network-specific neuromodulation offer promising avenues both research and clinical practice. Moreover, since brain networks operate in correlated and anticorrelated patterns, the exploration of multinetwork stimulation represents a critical frontier in developing brain state-dependent therapies. Regardless, translational research in the field of neuromodulation is shifting from the manipulation of single cortical targets to a new focus on cortical and subcortical networks, and the use of individual fMRI or tractography volumes is rapidly emerging. The importance of network-based approaches is widely recognized, but its implementation remains gradual and work in progress for the years to come.

Finally, we should keep in mind that the focus on the characterization and manipulation of anatomical and functional networks is only part of the equation and may simply represent the 'tip of an iceberg' recordable with non-invasive brain mapping approaches currently available to us such as EEG or fMRI. Indeed, although out of the scope of the present dissertation, delving deeper into the cellular and neurochemical local and network mechanisms subtending short-term and long-term plasticity will become increasingly critical for the design of appropriate stimulation strategies and technologies for which the priming, real-time monitoring and modulation of brain-state is crucial for effectiveness. This challenge, which includes for example the use of behavioural activities and neuropharmacology as priming or co-adjuvant tools to maximize not only the magnitude but also the region-, system- and cognitive domain-specificity of NIBS effects will be to our understanding driving the development of novel brain-state-dependent protocols, with unprecedented potential. Protocols in healthy participants and patients taking into account these factors are increasingly refined by tailoring stimulation to the real-time activity of cortical targets and associated networks, with preceding, ongoing or ensuing EEG/fMRI monitoring or with EEG-NIBS close-loop online approaches. Moreover, as monitoring technology evolves and is made more autonomous, lower cost, therapeutic applications based on immediately ongoing activity patterns (fluctuating over milliseconds/seconds) and longer-term brain state assessments over days, weeks or months (from acute to chronic phases post-stroke recovery process) will be soon embedded in any NIBS clinical session to ensure a more personalized flexible and effective management of stimulation protocols.

6. CONCLUSIONS AND FINAL REMARKS

The work presented in this dissertation can be framed as contributing the characterization of the physiological causal mechanisms subtending in the healthy and the pathological human brain the effects of weak electrical currents delivered to the primary motor cortex either with Transcranial Magnetic Stimulation (TMS) or transcranial Direct Current Stimulation (tDCS). According to the long term goal of this dissertation aiming to develop the use of weak magnetic and electric fields for the modulation of networked neuronal activity, Our findings represent an important leap from rodent models to human experimentation, advancing the understanding of how weak magnetic fields interact with neural circuits and providing new evidence for networked neuromodulation strategies suitable for clinical interventions. This research not only brings forth new insights into brain physiology and mechanisms of actions of TMS neuromodulation but also open up potential avenues for clinical applications in neuromodulation therapies.

The first objective and short-term goal of this PhD dissertation was to develop, implement and validate new analytical tools for TMS-EEG datasets to improve the localization of NIBS evoked activity on EEG datasets. We aimed to enhance the spatial and temporal precision by which we can characterize NIBS evoked physiological processes, such as an effect on normal or aberrant oscillatory activity and local/network synchronization, which play a mechanistic role in brain function. MRI based biophysical current distribution computational models and validation experiments strongly suggest that the used of individual MRI based E-field modelling for EEG spatial filtering allows a more reliable and specific extraction of neural sources and signatures, setting a more accurate framework for understanding the causal effects of brain stimulation on healthy or pathological brain systems and their associated functions and behaviors. Beyond the analyses of all TMS-EEG datasets included in the chapters and manuscripts of this thesis, the TMS-EEG spatial filter procedures we proceed in this doctoral dissertation could be integrated in venues such as EEG-NIBS or in close loop systems.

The second objective of this PhD dissertation was to ascertain and characterize the effects of very weak magnetic fields delivered on the primary motor cortex in single TMS pulses, short rhythmic TMS bursts on EEG correlates of *online* neuronal activity through TMS evoked potentials (TEPs), evoked ERSP and ITPC changes, as signs of entrainment and cross-frequency phase dependent effects. We also assessed the intensity-dependent effects of the former from very weak to conventional intensity and the ability of repetitive TMS patterns to modulate lastingly *offline* intracortical and cortico-spinal activity. Despite well-established uses of conventional TMS, the effects of weaker magnetic fields -tested in rodent models and in the human primary visual cortex- remained poorly tested in human motor systems. Our experiments conclude that very weak and weak magnetic pulses structured in short 20 Hz bursts induce ‘brain-state’ (phase-) dependent changes in ongoing oscillatory activity, and short-lasting *offline* effects when delivered repetitively at 10 Hz. All together, they also provide indirect evidence in favor of an effect mediated via intracortical inhibitory interneurons. Our outcomes contribute to strengthen the relevance of weak pulsed TMS fields in future therapeutic approaches and facilitate the development of TMS approaches suited for network manipulation.

The third and last objective of this PhD dissertation was to investigate the effects of single site vs dual-site anodal transcranial direct current stimulation (tDCS) on motor recovery following stroke. More specifically, we carried out a pre-registered clinical trial, E-Brain, in which we modulated the activity of two stroke-spared regions such as the contralesional dorsolateral prefrontal cortex, the ipsilateral cerebellum, or both combined. The rational being that a strategy operating on networks facilitating motor function via facilitation sustained attention/reward/planning and/or visuo-motor adaptation and learning might provide satisfactory outcomes. Indeed, most past neuromodulation strategies for stroke have focused on a single brain site, notably ipsileional or contralesional M1, with limited multi-network action and synergistic engagement of other systems. Hence, the approach was designed to target diaschisis-affected neural networks and foster the reactivation of residual connectivity between primary motor and cognitively contributing regions and employed standard MRI-based computational strategy to optimize tDCS montage on the different stimulated sites. Our data suggest that patients were compliant with the protocol and that all three active tDCS interventions were well-tolerated. Due still incomplete recruitment, we cannot conclude before a higher number of patients is completed. Nonetheless, preliminary isolated data suggests that combined dual site stimulation may enhance motor function and promote adaptive plasticity. This allows to, cautiously conclude that multi-focal brain stimulation targeting complex, large-scale network dysfunction may hold promise in stroke recovery.

In summary, our work contributes to the field of human neuromodulation in three different ways. **First**, we developed novel TMS-EEG analysis tools that superseding conventional approaches may increase the spatial precision and accuracy by which the effects of non-invasive brain stimulation can be ascertained on EEG recordings minimizing peripheral tactile auditory contamination by reentry signals. **Second**, our work highlights for the first time the potential of very weak to weak magnetic pulsed fields modulating evoked cortical activity or entrained oscillatory brain activity and phase dependent effects on the primary motor cortex of awake humans either used in single pulses, short burst if rhythmic TMS or repetitive TMS trains. **Third and last**, our clinical work in stroke patients with motor impairments provides evidence on the excellent tolerability and safety of a novel anodal tDCS-based strategy targeting non purely motor-execution regions (DLPFC and/or Cerebellum) contributing cognitively to voluntary motion, in attendance of completing the study in a higher number of stroke participants,

The present doctoral work addresses a few relevant questions while contributing to open discussions in the field of non-invasive brain stimulation, as much as it opens a myriad of new questions and challenges integrated in the evolution and progress of the domain. Future research should investigate the molecular and functional mechanisms of plasticity relevant for brain stimulation outcomes that underpin individual recovery trajectories in post-stroke movement disorders strongly considering inter-individual human variability, investigate cell-specific stimulation effects, adaptive networked neuromodulation opportunities, brain-state dependent neuromodulation and ultimately develop personalized therapeutic neuromodulation strategies. Indeed, despite nearly four decades of interesting technical developments, thrilling research and exciting applications, the field of non-invasive brain stimulation and neuromodulation is about to start a new exciting cycle. Besides unexpected surprises future will bring, the advent of new *procedures* (computational modelling and optimization, multimodal

imaging/mapping, analytic tools for NIBS-EEG and NIBS-fMRI, multisite stimulation, EEG/behavioural Close loop NIBS etc.), *novel technologies* (Transcranial Ultrasonic Stimulation, TUS, Transcranial Time Interference Stimulation tTIS for subcortical sources etc.), crucial *evidence* (interindividual variability, customisation, network manipulation, role of synchrony and synchronization, need for more efficient therapeutic interventions) and also *novel practices* (open access science, open data, collective multicentric open initiatives, collective shared datasets) will setting the agenda for the next 40 years to come. Certainly, the topics explored, investigated, and discussed in this dissertation will act as a *stepping stone* to embrace wider challenges within the field of neuromodulation and brain stimulation, setting the stage for the experimental and clinical adoption of weak electrical and magnetic fields to modulate human brain oscillatory rhythms and plasticity across complex brain networks.

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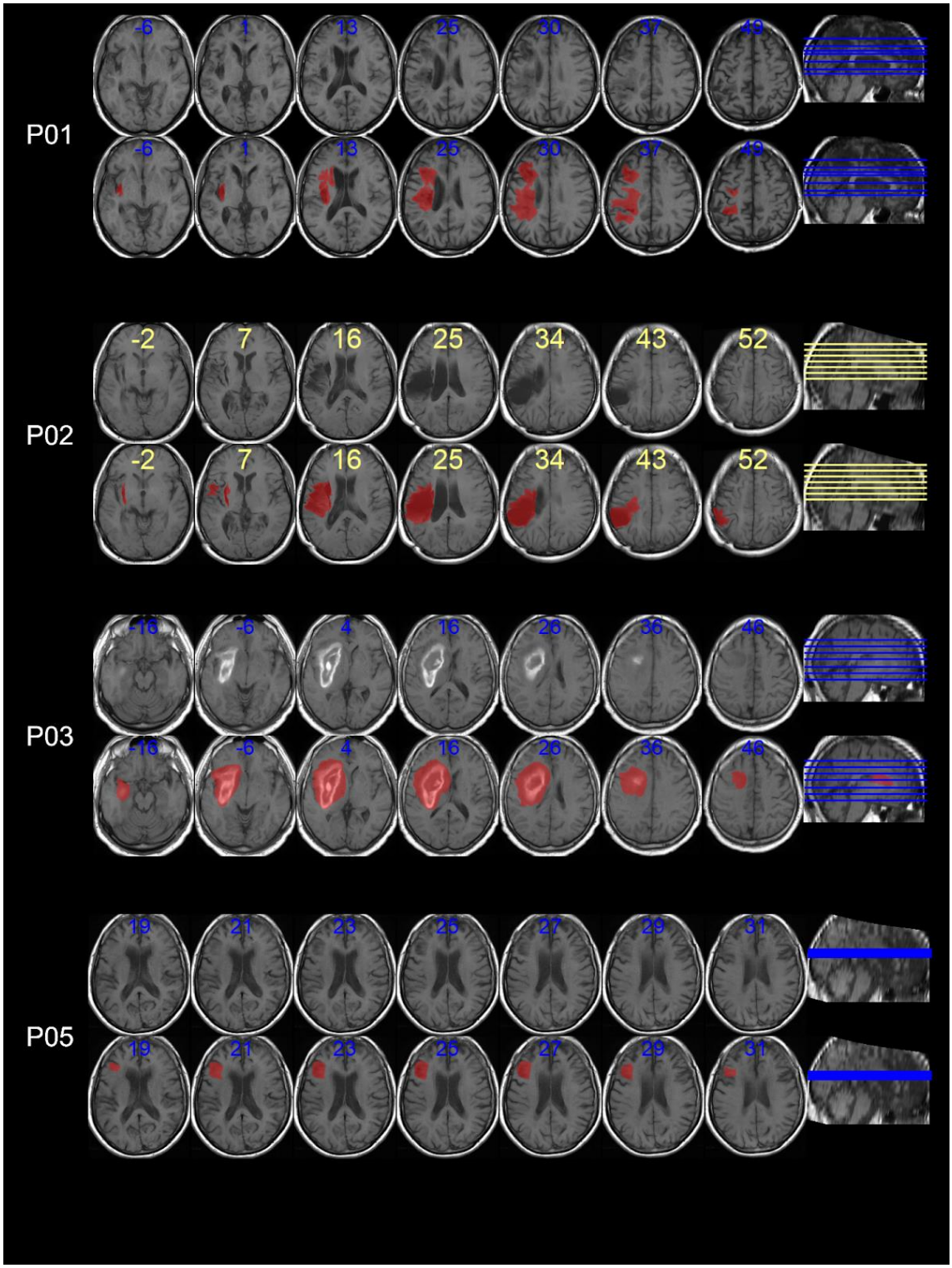
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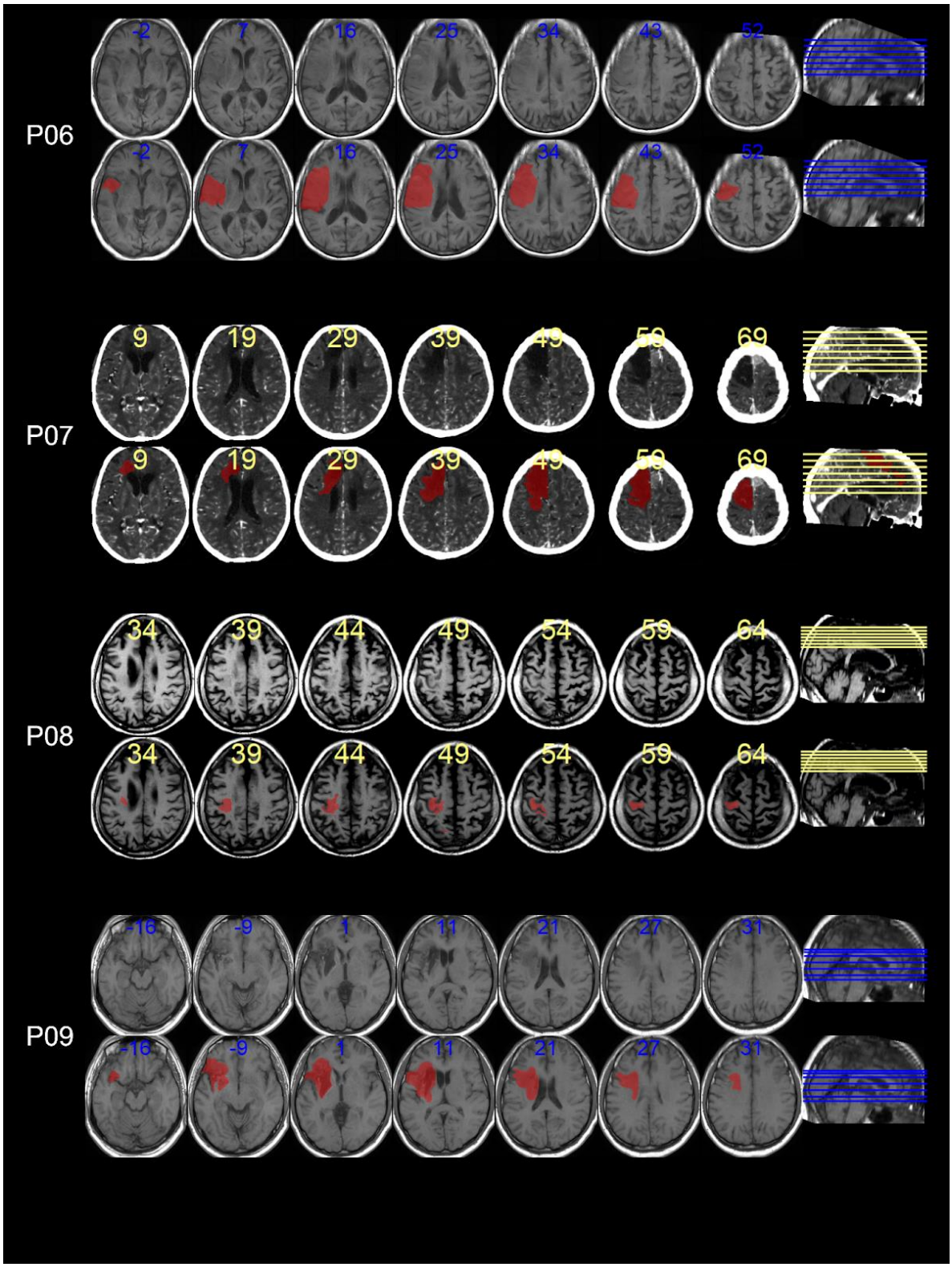
8. ANNEX

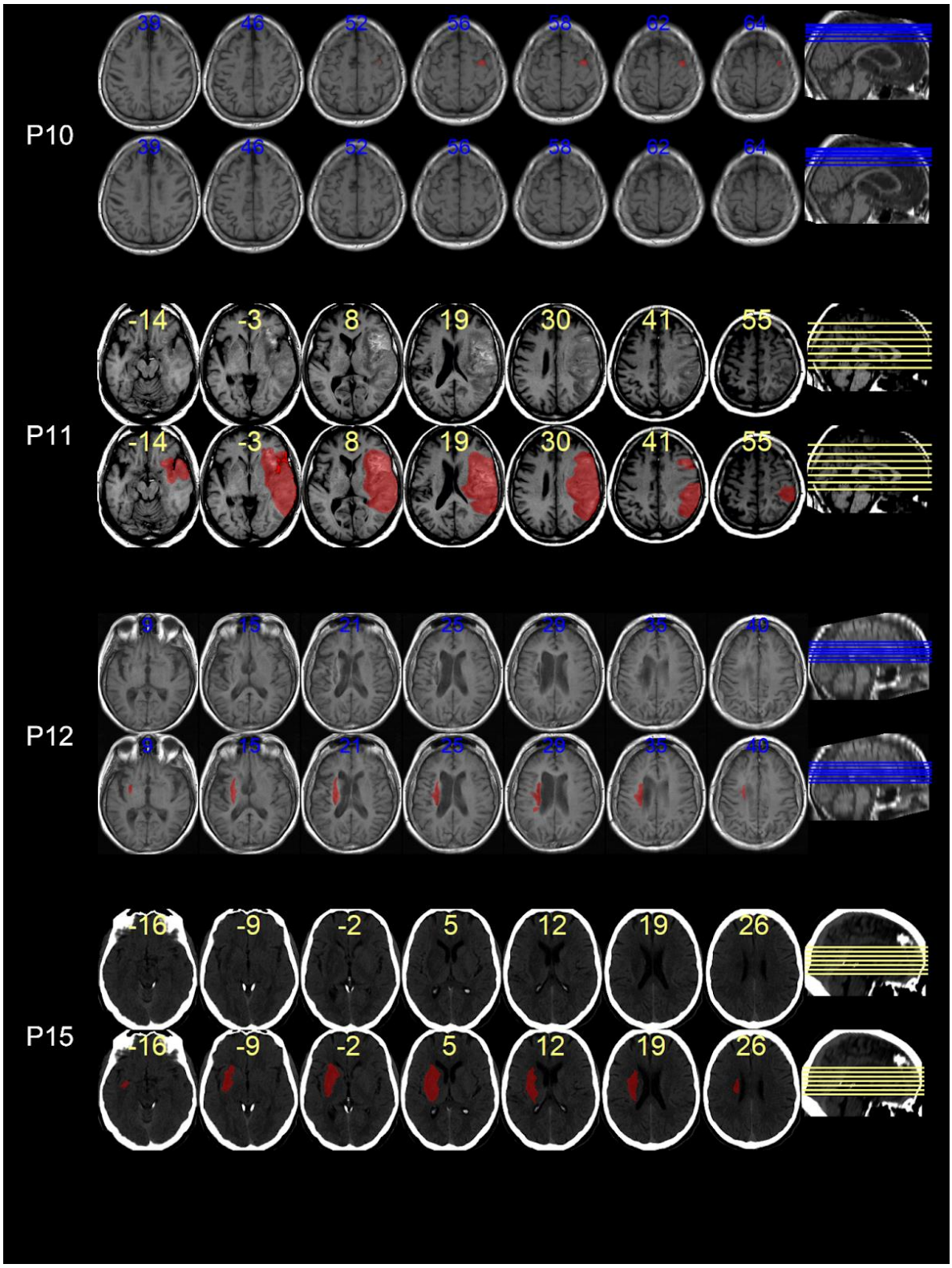
8.1. Manuscript 7: Supplementary materials 1

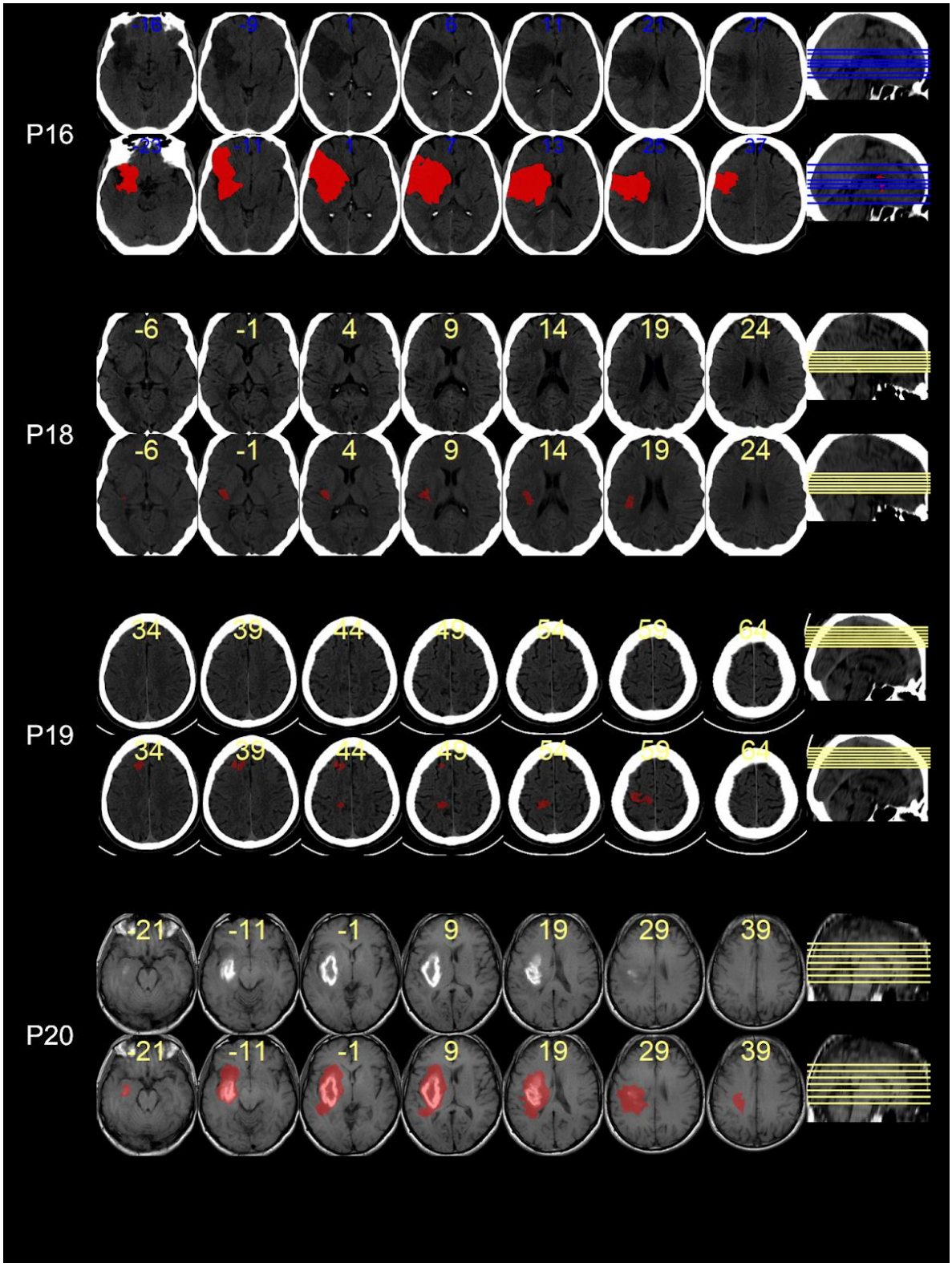
Supplementary Materials 1. Single subject lesion descriptives for all available MRI (n=23).

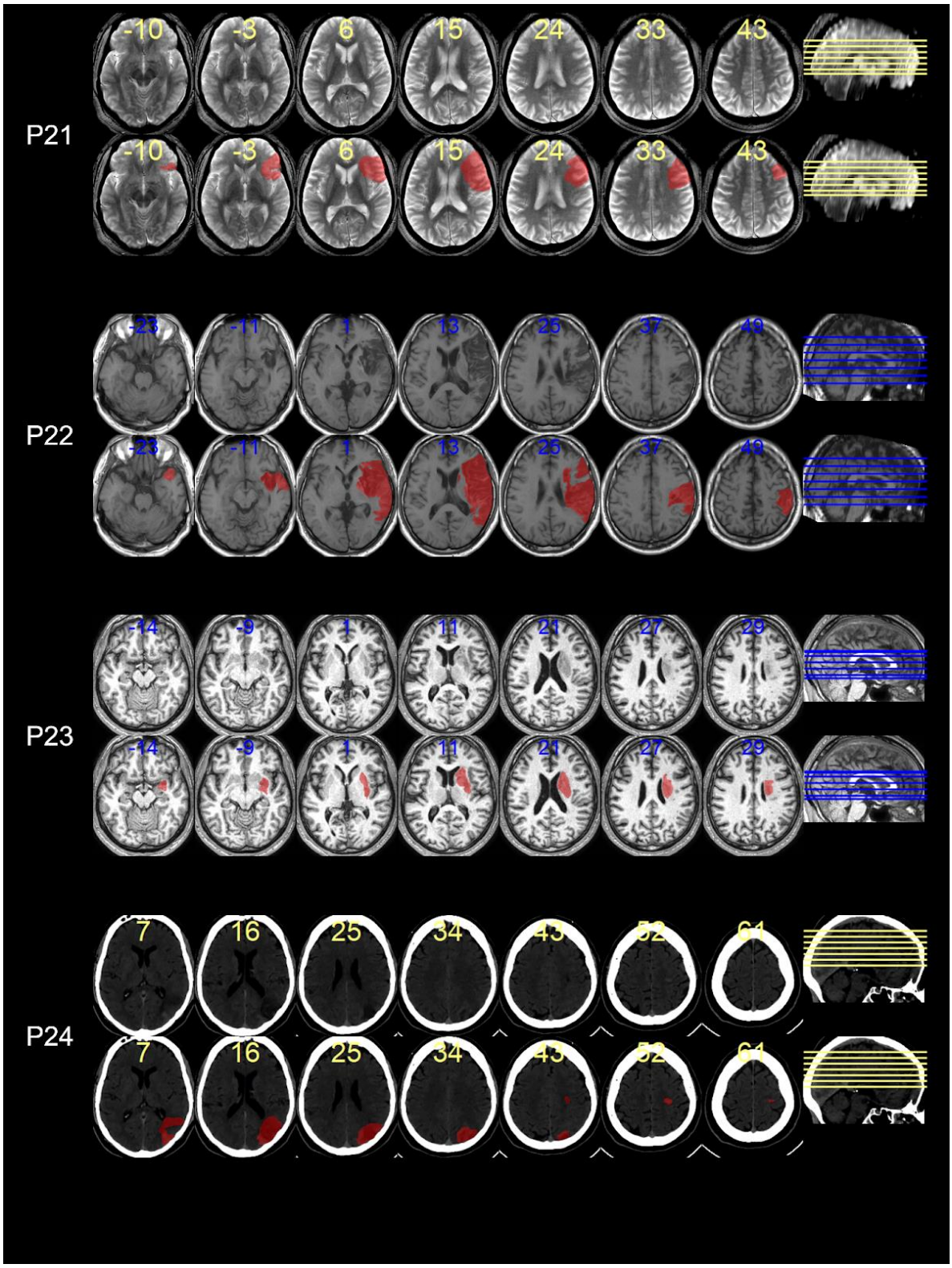
A. Single subject lesions (native space)

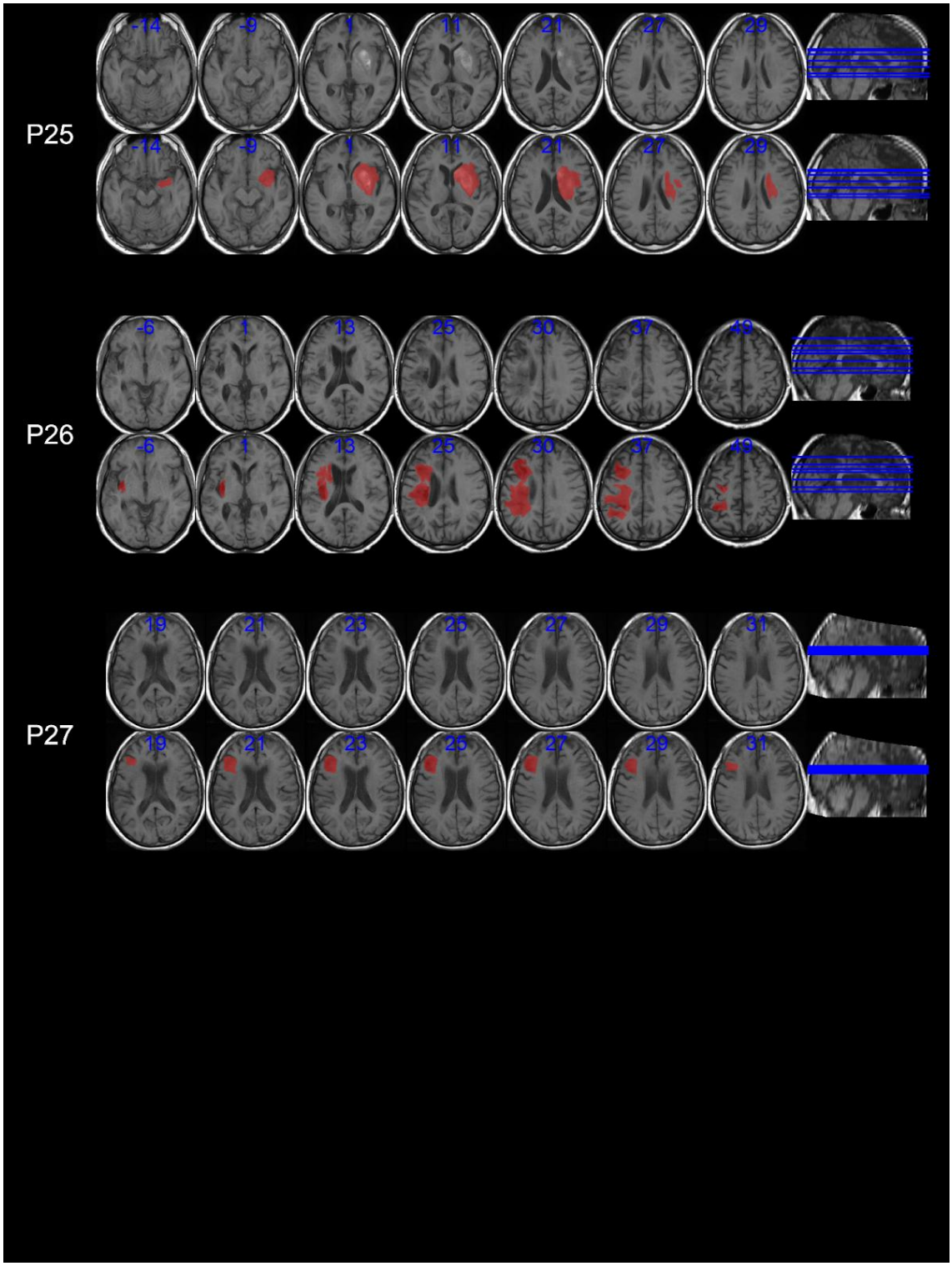



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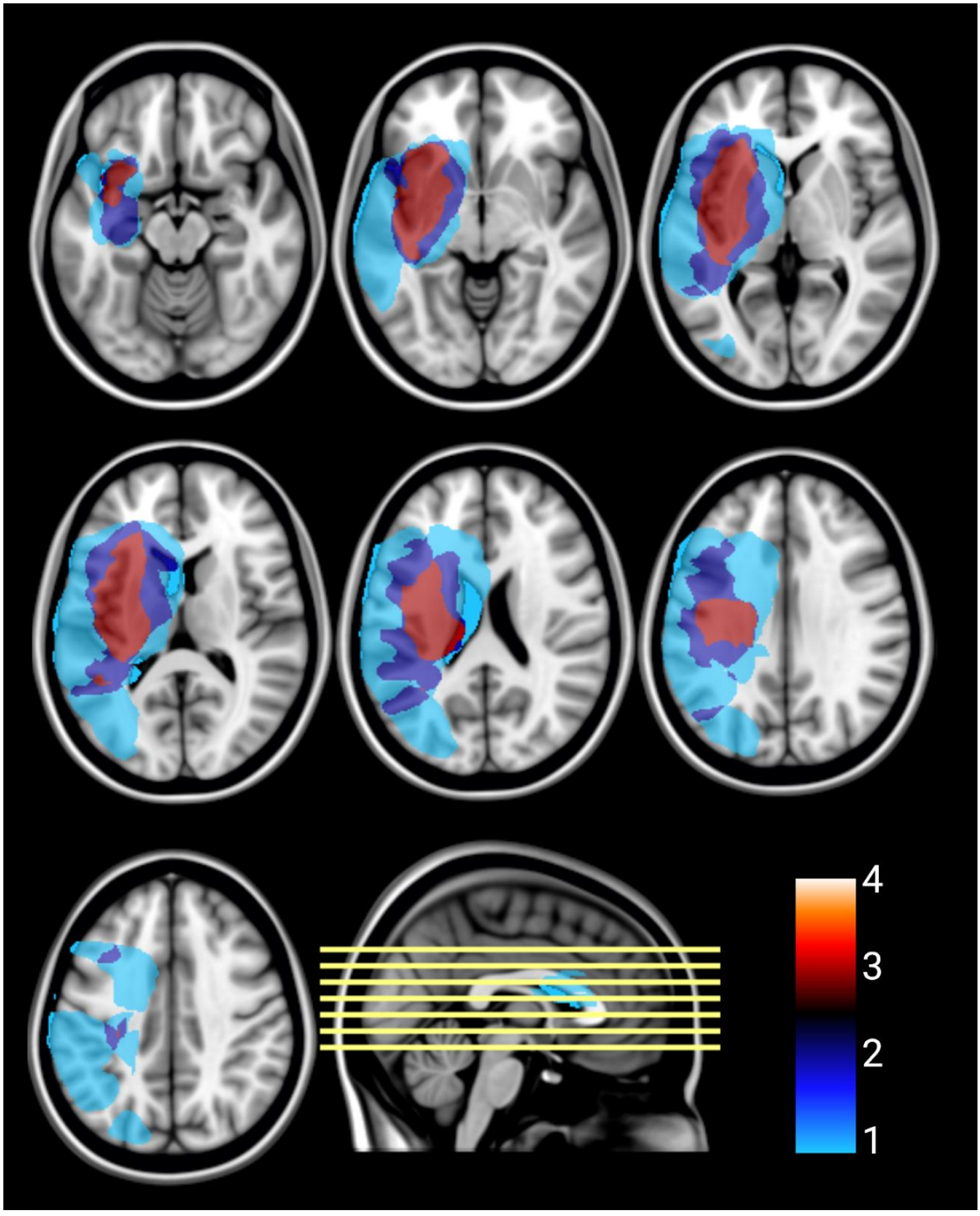

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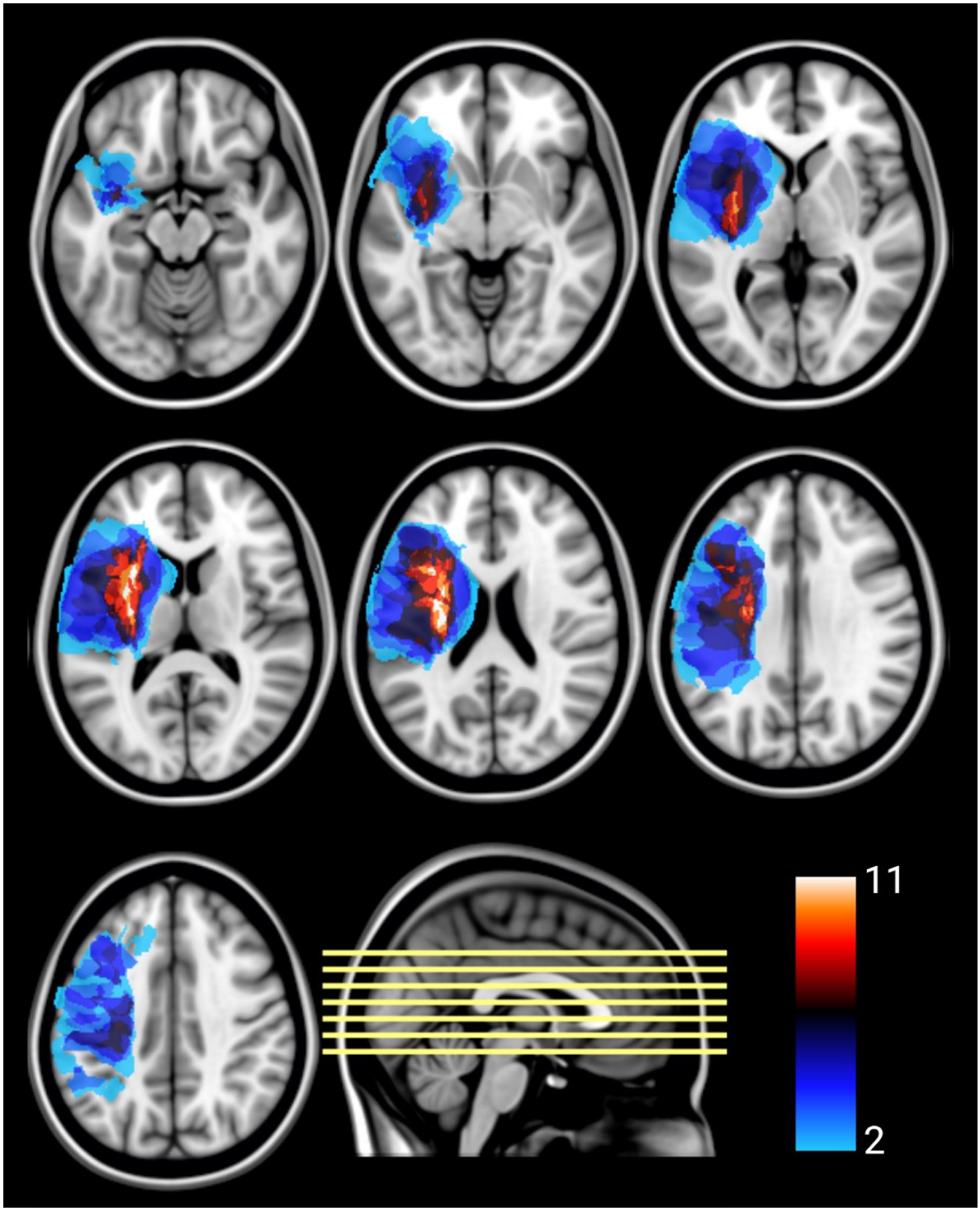

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B. Topographical overlap map of hemorrhagic lesions (n=4)



C. Topographical overlap map of ischemic lesions (n=19)



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Novel insights on the impact of very weak and conventional electric and magnetic fields by transcranial neuromodulation technologies: from low level neurophysiological effects to the rehabilitation of stroke patients
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