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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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 which has the potential to impact multiple cognitive and behavioral domains. Over the last 20 years, non-invasive brain neuromodulation via electrical (tCS) stimulation has shown the potential 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 trials.

Methods

We here present a double-blind, sham-controlled, single-center, randomized clinical trial involving participants having suffered a unilateral middle cerebral artery (MCA) stroke resulting in motor paralysis of the contralateral upper limb who will undergo a 10-days regime (5 days a week for 2 consecutive weeks) of a newly designed high-definition transcranial direct current stimulation (HD-tDCS) protocol. Clinical scale-based evaluations (e.g., Fugl Meyer, NIHSS, etc.), 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. In every stimulation session, participants will receive 20 min of high-density tDCS stimulation (HD-tDCS) (up to 063mA/cm^2) with a πcm^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 0.25V/m impact at each of the two 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 identify possible

interactions between the tDCS impact and motor outcomes. Our work will enrich our mechanistic understanding on prefrontal and cerebellar contributions to motor function and its rehabilitation following brain damage.

Trial registration

This study has been registered on ClinicalTrials.gov (NCT05329818)

Keywords

Stroke, neuromodulation, transcranial direct current stimulation, non-invasive brain stimulation, plasticity

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 associated subcortical structures. Despite the influence of lesion location or 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 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 resting membrane potential towards (anodal) or away (cathodal) from the neuronal firing threshold (7–10).

By virtue of such effects, single sessions of tDCS have shown the ability to transiently modulate cortico-spinal excitability, whereas periodical sessions 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 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 are often 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 location, extent, a large variety of stimulation parameters (electrode location, current intensity, density, regime periodicity, etc.), and interindividual head and brain anatomical differences influencing the distribution of tDCS-generated electric field (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 affected primary motor systems or the downregulation of homolog motor networks of the spared contralesional hemisphere linked with the former via inhibitory transcallosal interhemispheric projections. Nevertheless, new evidence regarding the neural substrate of motor paralysis and novel insights for restorative approaches

considering network reorganization capabilities provide solid ground on which to design new clinical interventions and test their efficacy. Also importantly, novel open-access tools allow simulating the distribution of electrical current fields (E-field) generated by NIBS taking into account head/brain structural features and tissue biophysical properties can be used to identify in brain computational models, optimal parameters and strategies maximizing E-field magnitude in single or multiple cortical sites considered important for recovery.

Among many factors driving novelty, the neural signature of MCA lesions has revealed strong interactions between alterations of sustained attention and large-scale desynchronization as factors limiting motor function or precluding recovery after brain damage (20). More specifically, the severity of motor deficits has been associated with the strengthening of impaired interhemispheric functional connectivity between the dorsal attention network (DAN) and sensory-motor networks (SMN), a reduction of anti-correlated activity between the DAN and the default mode network (DMN) (21–24) and high and low-frequency oscillatory abnormalities (decreases and increases respectively) in the injured hemisphere (25,26). Likewise, a connectome-based predictive model exploring fractional anisotropy (FA) in stroke has highlighted the role of ipsilesional prefrontal (DLPFC) and cerebellar areas subtending motor symptoms and their potential to convey motor recovery with NIBS interventions (27). Regardless, conventional therapeutic neurostimulation focused on directly modulating the excitability of damaged areas in charge of lost functions with inconsistent outcomes. For this reason, innovative approaches (28,29) attempt to reverse abnormal large-scale network signatures by inducing synergistic effects from spared cortical regions contributing to the damaged functions. We here claim that multi-site stimulation approaches that boost sustained attention and motor control systems by acting on key nodes of the networks subtending those functions might supersede the current clinical efficacy of traditional approaches (30–32).

We here obtained IRB approval to carry out a tDCS clinical trial (hereafter entitled *E-Brain*) based on the upregulation of accessible distant intact brain regions not directly involved in the fast control of motor activity (as it is the case of motor and premotor areas) holding interactions with such. More specifically, we will explore the potential of anodal tDCS over the dorsolateral prefrontal cortex (DLPFC) subtending sustained attention and cognitive control, the anterior lobe of the cerebellum (CEREB) involved in visuo-motor adaptation or the combination of these two targets in chronic MCA stroke patients.

Aims and objectives

Four main goals are pursued in this clinical trial: (1) We will identify the clinical potential of the multitarget stimulation by assessing the comparative effect of three tDCS interventions (cerebellar stimulation, dorsolateral prefrontal stimulation or the combination thereof) in driving improvements of upper-limb motor function in stroke patients; (2) We will 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 (cerebellum); (3) We will 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 interareal synchronization or changes of neural states abnormalities, taken as proxies of enduring adaptive plasticity); Finally, (4) We will identify biomarkers (clinical and cognitive scores, neuroimaging features and electrophysiological measures and tDCS-current distribution model parameters) associated to the severity of motor impairments and their improvement following stimulation.

In agreement with our goals, the primary outcome measure (addressing aim 1) of our trial will evaluate changes in the Fugl-Meyer Assessment (FMA) for the impaired upper limb. A set of secondary outcome measures taken prior to and following tDCS treatment (addressing goals 2, 3, and 4) will assess respectively: (2.1) patient's performance in a series of computer-based behavioral tasks evaluating sustained attention and cognitive control (prefrontal contribution) and visuo-motor adaptation (cerebellar contribution) performance, (2.2) changes in resting-state and task-evoked EEG recordings and (2.3) a 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.

The following series of predictions have been associated to the above-mentioned goals: (1)The modulation of cerebellar or dorsal-prefrontal systems, with active anodal tDCS -or the combination thereof- will improve motor function in stroke participants compared to sham tDCS stimulation (33). Moreover, a combined dual-site stimulation approach (prefrontal and cerebellar) will induce superior upper limb motor recovery respect to isolated interventions; (2) Clinical progress in upper limb motor function will be associated with performance improvement in computer-based tasks assessing dorsolateral prefrontal (attention and cognitive control) and/or cerebellar (visuo-

motor adaptation) contributions to motor function respectively; (3) The effect of prefrontal and cerebellar stimulation or the combination thereof will correct abnormalities in local and interareal functional connectivity (synchronization), which will be positively associated to clinical recovery. More specifically, we expect a strengthening of synchronization between the stimulated regions with premotor systems and increased interhemispheric integration restoring abnormal transcallosal balance (34,35). We also hypothesize that anodal tDCS will increase the variability of neural states, increasing neural complexity and plasticity capabilities (36); Finally (4) Isolated or combination of clinical, cognitive, neurophysiological and neuroimaging lesion features and tDCS current distribution model-based biomarkers will be able to predict lesion severity at baseline and response to tDCS treatment.

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 the hospital. MRI acquisitions will be performed in the *Radiology and Nuclear medicine* department of this same clinical institution.

Study design

The *E-Brain* protocol is designed as a double-blind, parallel, sham-controlled, randomized experimental clinical trial. 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; 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 gathered from the *Hospital Universitari Joan XXIII*, Tarragona (Spain) and retrieved from the participant's medical history before the

treatment onset. Clinical (scales or scores), behavioral (computer-based tasks) and electroencephalographic (EEG) evaluations will be carried out at 3 time points: I) Baseline assessment, 72h-96h (2-3 days) before the tDCS treatment onset; II) Post-tDCS assessment, 72h-96h (2-3 days) after the end of the treatment regime; and III) Follow-up assessment, 30 days after the end of the treatment regime. Across sessions, evaluations will be carried out under identical predefined conditions (see Fig.1A, B). The current protocol *E-Brain* follows the SPIRIT recommendations (see Additional file 1).

Fig.1. (Flow diagram and protocol items).

Participants recruitment

Participants will be enrolled trough the Rehabilitation and Physical Medicine department of the *Hospital Universitari Joan XXIII* in Tarragona, Spain. Only those participants 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 (R-MS) 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

We have established the following inclusion criteria 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 fronto-temporo-parietal territories); (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 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 an active treatment with sedative drugs); (2) Participants presenting contraindications to tDCS according to the most current international 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.

Exit criteria

Patients will be withdrawn from the study (1) if they manifest, at any time and without any need to provide any explanations, their willingness to stop their participation in the trial; (2) if they are not compliant with the procedures of the study; (2) if they experience severe discomfort or annoyance during their participation (i.e., insomnia, headache, etc.); (3) in case of unexpected events that incapacitate patients to continue in the study. We will include in the data analysis only datasets of participants that have completed at least 8 of the 10 stimulation sessions across the 2-week tDCS treatment.

Sociodemographic data

During baseline assessment, patients will be asked to complete to the best of their knowledge a questionnaire including the following information: (1) age and sex, (2) stroke features (hemisphere affected, localization, stroke type, time since episode, pre-morbid conditions), (3) socio-educational information (marital state, academic level, occupation, leisure hobbies, technology usage, sports practice, smoking, alcohol or drug usage, potential current medication), (4) past and ongoing stroke rehabilitation program (post-stroke-onset time, types of ongoing and completed programs, frequency, intensity, and periodicity). Lesion features will be completed with information participant's medical history accessed by an authorized clinician collaborating with the protocol (R-MS). Additionally, the Edinburgh manual dexterity scale and Beck's inventory will be administered to characterize the patient's laterality and assess the participant's mood, respectively.

Sample size

The current trial is designed as an exploratory clinical trial aiming to study the effects of an isolated monofocal (single site) or combined multifocal (double site) tDCS multiday intervention using a high-density array of electrodes. Given the exploratory character of our trial, no power calculation with a closed sample size was required by the local IRB committee. However, following recommendations of standardized guidelines for clinical experimental trials and considering the number of participants included in prior exploratory monocentric tDCS studies warranting sufficient statistical power (37), we aim to include n=15 patients in each of our 4 groups.

Randomization and blinding

The study will include n=60 patients with chronic MCA stroke who will be randomly assigned to one of the 4 experimental groups indicated above (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC+contra-CEREB, and SHAM). A patient randomization algorithm (*MinimPy* software running in *Python environment*, https://sourceforge.net/projects/minimpy/) counterbalancing groups by sex (Woman/Male), age (-65 years/+65years), and stroke type (ischemic/hemorrhagic) will ensure equivalence for those 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) (38) for sequential dynamic allocation where each new allocation is influenced by the current state of overall treatment balances (39). At the end of the study follow-up, investigators will debrief patients and ask them to guess to which group they believed they had been allocated (allocation perception).

Double-blind (both the participant and the investigators in charge of stimulation or evaluation 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 (MF, referred hereafter as the administrator) will program and blind the different HD-tDCS protocols programmed in our *Neuroelectrics Instrument Controller* system, whereas a second researcher, 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

condition) assessment performance. Exceptionally, blinding will be lifted upon request from medical and legal authorities in case of serious adverse events or upon reasonable demand by a participant after discontinuing participation.

Interventions

Active condition

HD-tDCS will be delivered with a *Starstim-8*® device, a wireless hybrid EEG/tES 8-channel system (Neuroelectrics, Barcelona, Spain). The *Neuroelectrics Instrument Controller*® software associated with this hardware will be used to pre-program all HD-tDCS and EEG protocols and carry out intervention sessions. Given the four-parallel arms characterizing the 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 participant's head circumference ensuring correct placement for NG pistim® electrodes (πcm^2 surface, Ag/AgCl) embedded in SignaGel® (Parker laboratories, USA) to keep impedances below 10 KΩ during stimulation. Every participant will receive 10 tDCS sessions of 20 min each, 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, depending on the experimental group different electrodes will 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. Additionally, during the stimulation sessions, the current intensity will be linearly increased (ramp-up) throughout the first 30 secs to reach: (1) 1.736mA intensity ($0.55mA/cm^2$ density) in the ipsi-DLPFC group (receiving ipsilesional prefrontal stimulation); (2) 1.999mA intensity ($0.63mA/cm^2$ density) in the contra-CEREB group (receiving contralesional anterior cerebellar stimulation); and (3) 3,735mA intensity ($0.55mA/cm^2$ density in the ipsi-DLPFC area and $0.63mA/cm^2$ in contra-CEREB) in the ipsi-DLPFC+contra-CEREB condition (receiving simultaneously dorsolateral prefrontal and cerebellar stimulation). Once reached the predefined

intensity, the current will be kept active for 20 min, a treatment duration that proved effective in previous studies in neurological patients (14,15). Finally, tDCS current will be ramped down along the 30s at the end of the session. Crucial values such as mean voltage, current intensity, and impedance of different electrodes employed will be recorded automatically during the sessions. Targeted regions are represented in Figure 2.

Fig. 2. (HD-tDCS targeted regions)

During stimulation, patients will be sitting in a comfortable chair. To keep patients awake and restrict interindividual and intraindividual variability caused by the diversity of neural states across subjects and sessions, participants will be asked to perform a simple computer-based game on a computer monitor placed 57 cm (arm's reach) in from of them requiring computer's keyboard presses (space bar) with their non-impaired hand, every time a moving dot contacts the limits of an 8 x13 cm rectangular placeholder. To monitor safety, and comfort and evaluate tolerance to the tDCS protocol, participants will be requested to complete before and immediately after each stimulation session, an adverse effect standardized questionnaire (10) documenting the incidence and intensity of the most common events such as itching, pain, burning, fatigue, headache or pain.

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 computational optimization was generated in MATLAB (R2019a, Mathworks, USA) and SimNIBS 3.2.3 (40), an open-source package for the simulation of non-invasive brain stimulation electrical field based on participant's MRI volumes using a standard head volume MNI152 (version 2009a) as a 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 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 (41,42): 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 best 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, and constrained by technical limitations of our ISO and CE certified tDCS device, a total maximal current of 2mA and a maximal individual electrode current of 2mA. 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) (43). For the computation of the optimal right contralateral cerebellum stimulation site (MNI x=-24 y=-66 z=-40) and optimally impact the anterior lobe of the cerebellum (contra-CEREB), the best solution was found with a total number of 5 scalp electrodes, a maximum current of 2mA and maximum individual electrode current of 2mA(44). We also modified the E-field *direction* controlling electric field strength at the target instead of a tangential mandatory 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 montage simulations excluded from being impacted.

Current evidence from *in-vitro* and *in-vivo* studies has demonstrated that tDCS intensities close to 0.25 V/m are sufficient to alter neuronal excitability via modulation of its resting state potential (9,45,46). 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 4mA injected current with high-density electrode montages (current density up to 0.63mA/cm^2), the optimization algorithm assuming a left hemisphere stroke (right ipsi-DLPFC and left contra-CEREB stimulation), suggested 0.25V/m simultaneously to both targets. This intensity was also retained to avoid possible side effects and warrant the safety/tolerability of the 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. 3 for details).

Fig. 3. (Computational biophysical models)

Sham 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 seconds, electrical current will be ramped up emulating the ipsi-DLPFC+contra-CEREB stimulation condition. Immediately thereafter, a 5-second ramp-down decrease will stop the release 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-second ramp-up followed by a 30 secs ramp down. 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 current intensity (47).

Associated clinical rehabilitation

In order to not 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' onsite rehabilitation program in our institution. Stimulation sessions will be conducted in the morning (8 am-12 am) and 'live' rehabilitation sessions will follow the stimulation session. The maintenance of rehabilitation activities during participation in the trial aims to maximize the chances of optimal recovery given the uncertain therapeutic value of our intervention and was specifically requested for ethical reasons by our local Institutional Review Board.

The on-site 'live' program is based on an intense multidisciplinary plan encompassing specific goal-directed qualitative interventions, combining physical therapy, occupational therapy, neuropsychology, and speech therapy interventions, with a periodicity of 1 to 2.5 hours a day, 2-3 days a week. All included participants will conduct equivalent rehabilitation activities based on the same goal-directed qualitative occupational principles. On stimulation days, when hospital rehabilitation cannot be performed 'live' and '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 reeducation. Occupational therapy exercises the fine motor function of the upper limbs, 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, depression, etc.).

Outcome measures

As indicated above, a set of clinical scales and computer-based tasks assessing motor and attention domains will be used to gauge the therapeutic potential of tDCS. Moreover, EEG will be measured and used to assess the impact on neurophysiological responses.

Fugl-Meyer Assessment (FMA)

Addressing the first and main goal of our clinical trial, the Fugl-Meyer Assessment (FMA) will be used to evaluate upper limb motor improvements. This is a widely used scale to assess motor impairment in post-stroke participants and is considered one of the most comprehensive and reliable quantitative measures for motor hemiplegic dysfunction (48). It provides an incapacity index divided into five sub-scales encompassing the assessment of functional motricity, sensibility, balance, joint range, and joint pain. Among the sub-sections of the scale, we will focus on the functional motricity assessment evaluating the upper-extremity motor domain (FMA-UE). Even so, the lower extremity section of the scale (FMA-LE) will be also administrated to assess the status and evaluate 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 three-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 signaling sound motor function; of these 100 points, 66 are attributed to the upper limb (FMA-UE) and 34 to the lower limb (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

To fulfil the second goal of our study we will rely on a visuo-motor adaptation task assessing motor learning, hence involving contributions from the anterior cerebellum to voluntary motor function. This task is an in-house computer-based paradigm designed to explore cerebellar contributions programmed in a MATLAB environment (R2019a, Mathworks, USA) and Psycho-Toolbox. Similar paradigms have been previously used to explore the human motor underpinnings of error control, skill learning, motor acquisition and/or adaptation and how the cerebellum interacts with such processes (49–52) with implications for neurorehabilitation. 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 limb. Their forearm will be supported in a thermoplastic pad minimizing gravity fatigue effects and ensuring a consistent semi-pronated arm position with 70º shoulder extension and 120º elbow flexion as the starting point for all evaluations. Additionally, hand splints to ensure correct attachment to the joystick will be employed if necessary and used 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 (1º). Participants will be asked to complete a series of consecutive trials to drive the cursor (a red dot) from the center of the screen (starting point) towards the interior of a randomly allocated peripheric 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 (5º) distance from the starting point. Participants will have 10 seconds to move the red dot and place it inside a circle and maintain such position for 0.5 seconds. Once completed, the green circle will jump back to the starting point, forcing participants to place the dot in the starting position to complete the trial. During the inter-trial interval (1.5 seconds) a full grey screen with a center stimulus ("+") will be displayed to keep participants vigilant. If the trial is not successfully completed and the participant does not locate 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 circles will be displayed in real-time (see Figure 4.A).

The task will be divided into 2 consecutive blocks. The first block (familiarization) will consist of 32 trials, whereas the second one (motor adaptation) will include 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 introducing a constant 45º angular rotation between the red dot movement and the actual joystick movement, deviating the red dot trajectory and forcing participants to compensate for such shifts in order to reach the target, hence show visuo-motor adaptive skills.

AX- continuous performance task

Also addressing the second goal of our study, we will also implement the AX continuous performance task (AX-CPT). This 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 such as sustained attention and cognitive control subtended by prefrontal systems. Moreover, performance in the task has been already correlated with the severity of motor impairment (53–55).

In this paradigm, participants comfortably seated at a distance of 57 cm from a 15-inch computer screen in an isolated room with no distractions, are 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'*, etc.). Participants are required to respond as quickly and accurately as possible after the cue-probe letter combination presentation during the intertrial interval with their non-impaired hand (please see Figure 4.B). 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 intertrial 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 the practice period while the task consists of a total of 150

experimental trials. Responses in both, the practice and the experimental blocks will be recorded, but only the latter will be used for statistical comparisons.

Fig. 4. (Computer-based tasks)

EEG acquisition

To address the third and fourth goals of our study, EEG, structural MRI recordings and a battery of clinical scales and cognitive assessments will be obtained. EEG data will be recorded with the same HD-tDCS used for stimulation, a *Starstim*® 8 EEG channel device controlled by the *Neuroelectrics Instrument Controller*® software (Neuroelectrics, Barcelona, Spain) able to sample scalp EEG signals at a frequency of 500Hz. EEG scalp NG pistim® electrodes (πcm^2 Ag/AgCl) and SignaGel® (Parker laboratories, USA) will be used for recordings. 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 pre-fronto-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 the visuo-motor adaptation cerebellar task and during the AX-CPT paradigm assessing dorsolateral prefrontal contributions to attention and cognitive control. Finally, 5 minutes 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 (56). It explores consciousness level, visual field surface, language function, the presence of hemineglect, hemiplegia, movement disorders, and sensory

function. It is made 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 use it to verify the comparability of each of the 4 experimental 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 (57). It is made by a series of questions and tasks specifically designed to assess visuospatial function, executive processes, working memory, and short-term memory, attention, concentration, language, and orientation. It contains a total of 10 items, for a total of 30 total points, where lower punctuations correlate with a higher level 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 control the cognitive impairment/improvement of our participants and asses group comparability.

Screening of hemispatial neglect

In order to monitor the presence of hemispatial neglect, participants will conduct a letter cancelation test, a bell's cancelation test, and a line bisection test. The letter cancelation test quantifies the presence of visual neglect scanning deficits (58). 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 cancelation test quantifies visual neglect deficits in the extra personal space (59). A total of 35 bells are embedded within 280 distractor figures presented in paper format. Participants will be instructed to encircle or cancel off all drawings corresponding to bells. All stimuli are displayed in black color over a white background of the same size. Though they might appear randomly distributed objects are presented in 7 columns, 3 columns on the left and right hemifields and one in the middle, with 5 bells and 40 distractors on each one. The

sheet of paper with the test 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 quantifies spatial neglect deficits (60). In this task, several examples of two types of black lines, 5 cm and 10 cm long, are presented on a white sheet placed in the middle of the participant's visual field. Participants are instructed to signal the center of each horizontal line and bisect with a pencil and are free to explore with the gaze stimuli.

MRI Imaging acquisition

MRI structural scans will be obtained from all stroke patients either drawn in anonymized from existing hospital clinical databases by an authorized neurologist working for the protocol, or if not available, will be recorded de novo within the two weeks before tDCS treatment onset. A 3 Tesla MRI scanner (Siemens Healthcare, Germany) located at the neuroradiology department at *Hospital Universitari Joan XXIII* will be used for image acquisitions. T1-weighted 3D anatomical images will be acquired with the following parameters: repetition time=2,500ms, echo-time=2.12ms, 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˚.

Data collection and management

All data generated by the study will be extracted and hosted in a secured internal virtual server, property of the *Universitat Rovira i Virgili* in Tarragona. A shared project with external encryption has been implemented allowing access to anonymized datasets to authorized investigators through a personal ID. The clinical trial will generate three large blocks of data: (1) demographic characteristics, (2) clinical outcomes, and (3) neuroimaging data like MRI scans and EEG recordings. Two computers property of the university with personal user encryption will be used to assist in data collection. Given the high volume and complexity of the pre-processing and analysis of MRI and EEG data, these outcome measures (raw data) will be only stored in computers and handled by expert investigators associated with the project. All data will be collected in the same hospital, and all co-investigators will be trained to respect the established data 'cloud' management protocol.

To maintain anonymity at all times, an ascendant number will be assigned to each participant at inclusion, identifying his/her data in the databases and representing anonymously the participant until the study is terminated.

Data analysis

Clinical and cognitive performance outcome measures

Clinical 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 to induce upper limb motor recovery following stroke. Mean changes post vs. pre-tDCS intervention in clinical scores, as well as during the follow-up assessment, will be compared across groups. Sociodemographic data will 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 patient's motor performance in the visuo-motor task (i.e., their ability to adapt to external perturbations) and assess tDCS effects to voluntary motion when applied over the cerebellum or prefrontal structures, a set of measures will be used: (i) the angular trajectory error ("SumErr"; cm) which estimates patients' accuracy and consists of the sum of observed deviations from the ideal linear trajectory at peak tangential velocity (PV) of each of the 64 trials during the motor adaptation block; (ii) the area under the curve (AUC) which measures patients' adaptation rate and is calculated by performing a power fit on the mean learning for each participant; (iii) the coefficient of variation (CV) which measures the variability of motor performance during the adaptation plateau phase, (i.e. when the AUC is stabilized and reaches plateau levels) and is calculated using the standard deviation (SD) divided by the mean over trials belonging to the plateau phase (AUC/Time-to-target).

In order to quantify dorsolateral prefrontal systems with the AX-CPT task, we will consider the total number of errors as a proxy of sustained attention failure. In order to quantify selective attention abilities, the number of outlined letters or bells and the deviation (in mm and % of the total line length) of manual bisections from the center of each line (letter and bell's cancelation and line bisection tests, respectively), will be used to estimate visuo-spatial performance. Other unanticipated complementary metrics could also be taken into account for the final analyses.

EEG data analysis

EEG offline pre-processing, artifact removal and the subsequent analysis will be performed using MATLAB (R2019a, Mathworks, USA), EEGLAB (v2021.0), and FieldTrip toolbox. Data will be preprocessed using a hierarchically organized pipeline proceeding as follows (61). For analyses of EEG during specific conditions, notably, eyes-open resting-state recordings prior to and following treatment or prior to and following each tDCS daily session (visuo-motor adaptation task and the AX-CPT task) the same procedure will be followed. EEG data will be segmented into contiguous epochs after a preliminary investigation on what epoch length could be the most robust for the analysis. We will apply a 2nd order infinite Butterworth forward and backward filter with a 0.5-45Hz low-pass high-pass filter at a resolution of 2Hz (500 ms time windows) with a Hanning taper window to minimize leakage. In case of unexpected noise, secondary notch filters will be used to further clean the data. Data will be visually inspected a first time to identify noisy channels whereas eye movements or muscle-related artifacts will be removed using a common Independent Component Analysis ('runica'), supervised and manually corrected after expert visual verification. Epochs exceeding 120μv peak-to-peak amplitude signals will be also removed. Finally, channels will be re-referenced to their common average, and data will be right-inverted always representing the neuronal activity of the damaged hemisphere on the right side. This will allow reliable group comparability independently of the damaged hemisphere.

After data pre-processing, (i) power spectrum density (PSD), (ii) connectivity, and (iii) complexity analysis will be carried out. Other metrics (e.g., EEG microstates) may be also considered for analyses. All these approaches will be individually conducted (for each subject) for every condition (resting state recordings, visuo-motor task, AX-CPT task) at every registered time-point (baseline, post-TDCS, follow-up) in order to perform further statistical comparisons.

The spectral analysis will be conducted in order to explore pre-to-post-to-follow-up intervention 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 the Welch's method with a 500ms periodogram. Relative and absolute spectral normalized power (NP) will be then extracted for each electrode according to the following formula (equation 1):

$$\mathbf{NP= 100} \times \frac{\sum \boldsymbol{F}}{\sum \boldsymbol{Total}} \quad (1),$$

where *F* represents a specific frequency band, and *Total* the whole frequency spectrum (0.5-60 Hz). Normalized average power for all frequency bands will be computed and plotted to explore differences between pre-and post-tDCS and follow-up changes within groups and compare differences among tDCS modalities tested in independent groups.

Functional connectivity analyses will be conducted to explore tDCS-related 'reshaping' of intra-hemispheric, and inter-hemispheric connectivity (assessing functional integration and segregation mechanisms at the network level). For intra-hemispheric connectivity, our 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 analysis will focus on synchronization measures between premotor/M1 (electrodes C3-C4) and also prefrontal systems (electrodes F3-F4). Overall, an 8x8 connectivity matrix will be explored contemplating all connections between all possible electrode combinations.

Functional connectivity will be assessed by means of EEG coherence measures. Coherence has been shown to provide a reliable measure of synchronization between a pair of electrodes (phase and amplitude consistency difference correlation at a given frequency), hence identifying functional associations between underlying brain sources. Additionally, given 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*) (62) by means of a Fast Fourier Transformation (FFT). This specific synchronization measure is one of the most robust to estimate functional connectivity between a pair of electrodes (63) and is calculated as follows (equation 3):

$$\boldsymbol{ImCoh^{x,y}(f,t)} = \frac{1}{N}\sum_{K=1}^{N} \frac{F_k^x(f,t)\mathrm{x}F_k^y(f,t)^*}{\left|F_k^x(f,t)\mathrm{x}F_k^y(f,t)\right|} \quad (3),$$

where $ImCoh^{a,b}(f,t)$ represents the imaginary part of coherence between a pair of channels (x and y) of a specific frequency band *f* for a centered time window *t*. *N* is the number of trials and $F_k^x(f,t)\mathrm{x}F_k^y(f,t)^*$ represents the normalized cross-spectra between two time-series. Based on previously stated hypotheses and predictions, we will

investigate main *ImCoh* changes pre-to-post-to-follow up for inter-hemispheric and intra-hemispheric connectivity.

Complexity analyses will be conducted to explore tDCS-related changes of neuroplastic properties. In order to quantify EEG complexity, we will estimate the predictability of EEG signals following a given tDCS condition by calculating multiscale sample entropy. This estimate quantifies the probability that neighboring points may be in a predetermined rage in a time series $\{x_1, x_2, \ldots x_N\}$, computing how often patterns reoccur in a time-domain sample (i.e., temporal irregularity prediction of time-domain). The computation of Multiscale Entropy (MSE) is divided into two steps: (1) first, a moving-averaging procedure is computed to express the dynamic representation of a system by means of the following formula (equation 4):

$$\boldsymbol{Z_j^t = \frac{1}{t}\sum_{i=j}^{j+t-1} x_i}, \qquad (4),$$

where z^r represents the moving-averaged time series, t represents the scale factor and j $(1 \leq j \leq -t + 1)$ represents the time index, and (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 (64) for complete MSE mathematical formulation).

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 drown outlining damaged areas directly on the T1-wheigted 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 (XC-T) trained in neuroimaging and neuroanatomy upon advice from additional co-investigators (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 for every parcel and damaged tracts will be characterized and correlated with 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. Nonetheless, due to anatomical interindividual differences, the impact of electric currents at targeted structures (DLPFC and anterior lobe of the cerebellum) can slightly differ from subject to subject. For that reason, individual biophysical models will be generated to estimate E-field (electrical current fields) distribution on each participant MRI. On such basis, we will study *post-hoc* the influence of model-generated variables (peak E-field density 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 outcome measures.

To this end, T1-weighted MRI individual head models will be reconstructed with the automatized *headreco* functions in SimNIBS 3.2.3. Derived lesion-segmentation masks (air, bone, CSF, eyes, GM, WM, and skin) will be manually corrected by means of ITK-SNAP software (ITK-SNAP 3.8.0, http://www.itksnap.org) with a graphic tablet (WACOM One) by an experienced researcher (XC-T) upon advised by expert co-investigator (AV-C and MT). After manual correction, head models will be re-reconstructed to ensure correct lesion and mask limits delineation. Then, tDCS current distribution and target peak magnitude of the aforementioned electrode montages will be simulated in SimNIBS using finite-element modelling 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.

Electric fields for each subject will be transformed and warped to the MNI152 standard head model by means of *Freesurfer (v.7.2.0)*. Moreover, electric field strength (|E|) and the normal component of the electric field (|nE|) at predefined regions of interest (ROIs) corresponding to the stimulation target coordinates will be also extracted across participants directly from native space in SimNIBS, transformed to the normalized space and correlated with EEG and behavioral clinical outcomes. Spherical 5 mm diameter ROIs will be defined and the highest electric field value for |E| and |nE| obtained from every simulation.

Data extracted from individual models will be correlated to modeled peak currents on stimulated sites on 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, in-vitro and ex-vivo studies have shown a lineal correlation between subthreshold neuronal depolarization and electric field strength (9,65), therefore, we will correlate peak current density at cortical targets modeled individually with resting state EEG measures, informing on cortical excitability or the state and changes of interhemispheric, intrahemispheric and cerebello-frontal functional connectivity.

Statistical analysis

Prior to any comparison across tDCS stimulation groups (GROUP 1: ipsi-DLPFC, GROUP 2: contra-CEREB, GROUP 3: ipsi-DLPFC+contra-CEREB and SHAM) we will verify that the severity of motor impairments (Fugl-Meyer Assessment) at baseline is not statistically different between the 4 groups. Additionally, potential differences on socio-demographic equivalence (hemisphere affected, mean age, sex) will be also tested to ensure group comparability. To that end, one-way ANOVA or X^2 followed by the Pos-hoc Tukey test and Benjamini-Hochberg correction will be respectively performed to test homogeneity between groups.

In order to address the first goal of our study (primary outcome measure), which is to evaluate comparatively the therapeutic potential of three active tDCS strategies (GROUP 1: ipsi-DLPFC, GROUP 2: contra-CEREB, GROUP 3: ipsi-DLPFC+contra-CEREB) compared to SHAM stimulation in post-stroke motor recovery through the Fugl-Meyer Assessment, we will first asses if there is a main effect of the tDCS intervention across the follow-up. In a second step, we will address if the combined strategy (GROUP 3: ipsi-DLPFC+contra-CEREB) results in better outcomes than those achieved by GROUP 1 (ipsi-DLPFC) and GROUP 2 (contra-CEREB). The same procedure will be used to assess the second goal of our study (secondary outcome measure 2.1) and verify if similar differences exist with regard to the impact of stimulation on attention and cognitive control according to the AX-CPT task, and on motor adaptation using the visuo-motor adaptation task.

To this end, all data will be presented as mean ± standard deviation. Statistical significance will be set as $p \leq 0.05$ for all tests. Data distribution will be tested by means of Shaphiro-Wilk method. In case of normal distribution, clinical outcomes (see outcome measures section) 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. Pos-hoc pair-wise comparisons will be performed using a two-tailed (student's) *t* test with Bonferroni correction for multiple comparisons. In case of non-normal data distribution, a non-parametric Kruskal-Wallis test will replace repeated measures ANOVA. Linear mixed models and Pearson's coefficients corrected for multiple comparisons will be explored between AX-CPT, visuo-motor adaptation task, and the Fugl-Meyer assessment to explore any possible behavioral correlations between motor and attentional and cognitive control domains.

In order to assess the third goal of our study (secondary outcome measures 2.2) and characterize the electrophysiological EEG correlates induced by our interventions, we will explore correlations with behavioral data and biomarkers of recovery. First, power spectral density (PSD) changes for each frequency band will be compared between groups (ipsi-DLPFC, contra-CEREB, ipsi-DLPFC+contra-CEREB, SHAM) 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 (1.000 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) 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 (P=0.05) 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 total averaged connectivity and entropy values and individual topographical maps at the electrode level will be examined to detect specific regions sensible to intervention.

To assess the 4th and last aim (secondary measure 2.3) of this study, outcomes variability among induced E-field components will be explored. The individual main differences across the follow-up of the Fugl-Meyer Assessment evaluating motor recovery, cerebellar visuo-motor adaptation task and AX-CPT task assessing attention and cognitive control will be correlated (Pearson's correlation, p=0.01) 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 analysis. Finally, multivariate regression models will be computed to explore correlations between electrophysiological response (μV^2, *ImCoh* and entropy measures), induced E-field, and outcome behavior. All statistical analysis will be performed with the R statistical environment (v3.5.0), Fieldtrip toolbox, and MATLAB.

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 following a unilateral middle cerebral artery at the chronic phase of stroke. The novelty of our protocol is that instead of aiming to modulate damaged cortical areas or their contralesional homologs, it focuses on assessing the isolated or combined stimulation of two regions such as the ipsilesional prefrontal cortex (DLPFC) and the contralateral anterior cerebellum (CEREB) not directly involved (as premotor and primary motor systems are) in the execution of the motor activity but contributing indirectly via 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 up-regulation (with anodal tDCS, high-frequency rTMS or iTBS patterns) of ipsilesional M1/premotor systems, (ii) the down-regulation (with cathodal tDCS, low-frequency rTMS or cTBS patterns) of spared contralesional M1/premotor systems by virtue of the trans-callosal rivalrous interactions, remapping, reorganization or normalization of abnormal excitability of lesional/perilesional areas (66) or (iii) the up-regulation of motor regulation systems such as the supplementary motor area (SMA) or the cerebellum. Unfortunately, initial enthusiasm for many of these approaches based on small clinical trials has dwindled by the lack of consistent effects when tested in larger populations of patients, and efficacy remains debated (67). 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 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 rehabilitation of prefrontal functions such as sustained attention

and cognitive control (35,68,69). Likewise, the modulation of the anterior 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 (30–32). 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), and the need for restorative stimulation methodologies able to integrate large-scale network-wide synchronization mechanisms across these structures. However, the limitation of spatial resolution of clinical EEG with few recording sensors -useful to explore individual clinical evolution of neurophysiological markers in individual patients has limited our comprehension of the underlying processes following motor recovery in stroke. Moreover, technical limitations of the first generation of tDCS devices to implement multi-site stimulation (i.e., targeting with an acceptable spatial resolution with high-density montages of different cortical nodes simultaneously) have contributed to keeping network approaches poorly explored.

In such context, the protocol *E-Brain* aims at comparing in separate 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 modulation by means of tasks assessing sustained attention/cognitive control and visuo-motor adaptation skills respectively, to verify that ultimate upper limb motor improvements were 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 (70), and reveal the longer-term neuroplastic properties associated with such effects.

Still a key challenge for clinical neuroscience (73,74), 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 dysfunctions (71,72). Moreover, it is supported for example by recent work demonstrating higher modulatory power on cortical reactivity (69) and changes in corticospinal excitability (75,76) 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 motor but also non/motor cognitive systems contributing indirectly to voluntary motion. Under such perspective, we will pursue the 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 have tried to minimize. First, recovery of lost motor functions 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 significant motor skills reacquisition. Nonetheless, a more intense (i.e., a higher number of daily and cumulated sessions over time) and longer-lasting regime or follow-up is currently outside of the scope of our protocol and limited by the scarcity of available time and resources of investigators and also patients. Two weeks of stimulation and a month follow-up post-treatment seems a reasonable compromise for an experimental trial that once proven potentially successful we will develop at a larger scale. Second, motor stroke patients even if sharing the same cardinal symptom, upper hand motor paralysis, can be affected by different lesion types, volume and location, differentially impacting the severity of their impairments, their ability to fully understand and perform motor or cognitive tasks and compromise the reliability of behavioral or EEG recordings. Hence, even if our *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' onsite 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 our 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 and these will be regularly questioned with regards to their daily life activities habits during participation to the study and their comments documented.

In sum, the alarming pandemic-like rates reached by the consequences of acquired brain damage in developed societies (77) 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 attention and cognitive control by prefrontal networks and also to visuomotor adaptation and optimization by cerebellar systems. More generally, beyond pure clinical applications in MCA stroke motor recovery, our study is designed to provide insight on the anatomical and physiological foundations of motor impairments and tDCS neuroplastic phenomena driving the recovery. Finally, if our intervention demonstrates clinical efficacy, our protocol will pave the way for the development of individually-customized tDCS strategies based on multisite modulatory interventions at a larger scale and the design of more sophisticated and better-adapted tDCS technologies.

Trial status

The first version of the study protocol was approved on 9 July 2021 by the local ethics committee *Institut d'Investigació Sanitària Pere Virgili* (IISPV, Tarragona, Spain). Eight pilot subjects underwent successfully the current protocol between July 2021 and November 2021. Data pertaining to the N=8 pilots will not be included in the study. Participant recruitment began on March 2022; since then, 11 subjects have successfully completed the current protocol. Approximately, participant recruitment will be completed in December 2023.

Addition File

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.

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: grey 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, BSIdir: pair-wise derived brain symmetry index, PET: positron emission tomography, PSD: power spectrum density, ROIs: regions of interest, Sh: sham, 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.

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Dissemination policy

The findings of the current study will be analyzed by the authors of the present manuscript and published in international peer-review journals and presented in leading national and international conferences. Anonymized outcome measures datasets and codes for data analyses will be published in Online open access repositories. Results will be also communicated to health professionals and participants.

Authors' contributions

All authors read and approved the current manuscript. XC, MTC and AV-C wrote the first version of the manuscript, and improved it with comments by MB. 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's 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, MF, R-MS and MV-L will be in charge of tDCS interventions and evaluations. MB, BB and CG designed and adapt the cerebellar assessment computer paradigm and will contribute to analyses and its interpretation, whereas MT will be in charge of assisting with MRI-based stroke grey and white matter 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* (https://www.iispv.cat) will annually monitor and audit the evolution of the present clinical trial and approve its continuation.

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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.

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 *Institut d'investigació sanitària Pere Virgili* (IISPV, Tarragona, Spain) and is in accordance with the Declaration of Helsinki. All participants will be required to provide informed consent, which will be gathered by the main investigator XC. The present study is registered on ClinicalTrials.gov (NCT05329818).

Consent for publication

EEG, MRI, images or any other clinical details will not compromise participants anonymity.

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. A Flow diagram of the study design. The diagram is represented as a progressive weekly calendar where W0 denotes the baseline assessment and the interventional starting point for a representative included subject. cCEREB: anodal contralesional cerebellar stimulation, iDLPFC+cCEREB: anodal ipsilesional DLPFC combined anodal contralesional cerebellar simultaneous, iDLPFC: anodal ipsilesional dorsolateral prefrontal stimulation, SHAM: sham, 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 description and timing of enrolment, treatment and assessments during the study.

Figure 2. 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 lobes I-IV of the cerebellum anterior lobe (CEREB). **A** DLPFC target area (MNI coordinates X=-39, Y=34, Z=37) in axial (**a.1**), coronal (**a.2**) sagittal (**a.3**) and skin projection (**a.4**) views. **B** Cerebellum target area (MNI coordinates: X=-24, Y=-66, Z=40) in axial (**b.1**), coronal (**b.2**) sagittal (**b.3**) and skin projection (**b.4**) views. Target areas are represented in the MNI152 and labelled on a model standard MRI by means of an MRI-based frameless stereotaxic neuronavigation system for image acquisition (Brainsight).

Figure 3. Computational biophysical models of an in-house developed ipsilateral dorsolateral prefrontal (ipsi-DLPFC), anterior contralateral cerebellar (contra-CEREB) and combined prefronto-cerebellar (ipsi-DLPFC+contra-CEREB) tDCS montages using a 3D reconstruction of the MNI152 model brain developed in SimNIBS 3.2.3. Electrode positions surrounded by red circles represent active anodes, those surrounded by blue circles represent active cathodes, and 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 (1.736mA), F1 (-1.222mA) and FC5 (-0.536mA). **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): P010 (1.960mA), FT10 (-0.550mA), CP6 (-0.180mA), Oz (-0.630mA) and P9 (-0.631mA). **C** Final simulation merging DLPFC and cerebellar electrode montage supposing a left hemisphere stroke, pertaining to the frontocerebellar simultaneously 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 0V/m (blue areas) up to a maximum of 0.36V/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, nomE: electric field strength (|E|).

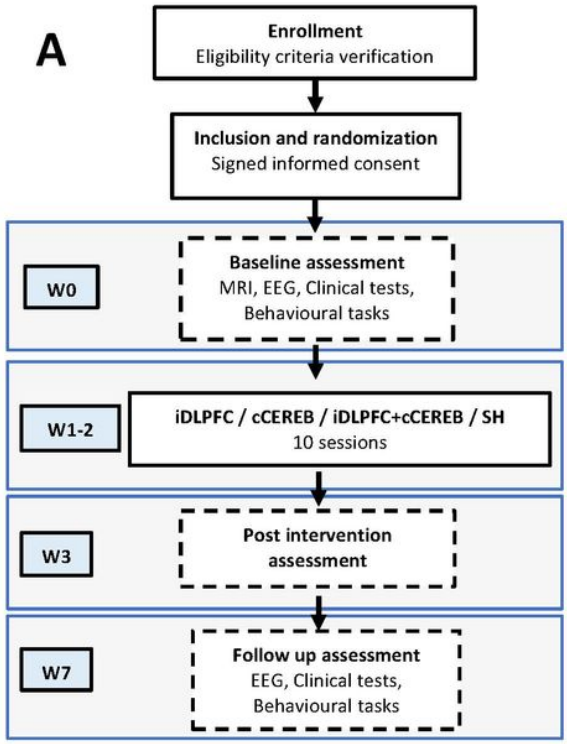
Figure 4. Computer-based selected tasks to assess cerebellar (motor learning and adaptation) and dorsolateral pre-frontal (sustained attention and cognitive control) contributions and their changes following stimulation. **A** Visuo-motor adaptation experimental paradigm design and setup. Following a stimulus ("+"), a red cursor representing the joystick position is displayed in the center of the computer screen. Participants are required to drive the cursor moving the joystick towards a random allocated target (during 10 seconds) displayed as a green circle equally spaced around a virtual circle. In the motor adaptation block, a force-field with a 45º constant perturbation is applied deviating the cursor movement respect the actual joystick movement. **B** AX-CPT experimental design.

Participants are instructed to be aware to a serial presentation of letters making a target response each time the correct cue-probe (A+X) combination is presented (press key '1' on a keyboard), or an alternative response (press key 'z' on a keyboard) for all other incorrect cue-probe combination (e.g., A + S, M + X, etc.). During the experimental recording, a total of 150 trials encompassing the presentation of a cue stimulus (1000ms), a fixation stimulus "+" (1000ms) and a probe stimulus (1000ms) followed by an inter-trial interval (1500ms) are completed. ITI: Inter-trial Interval

Additional Files

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

Figures



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

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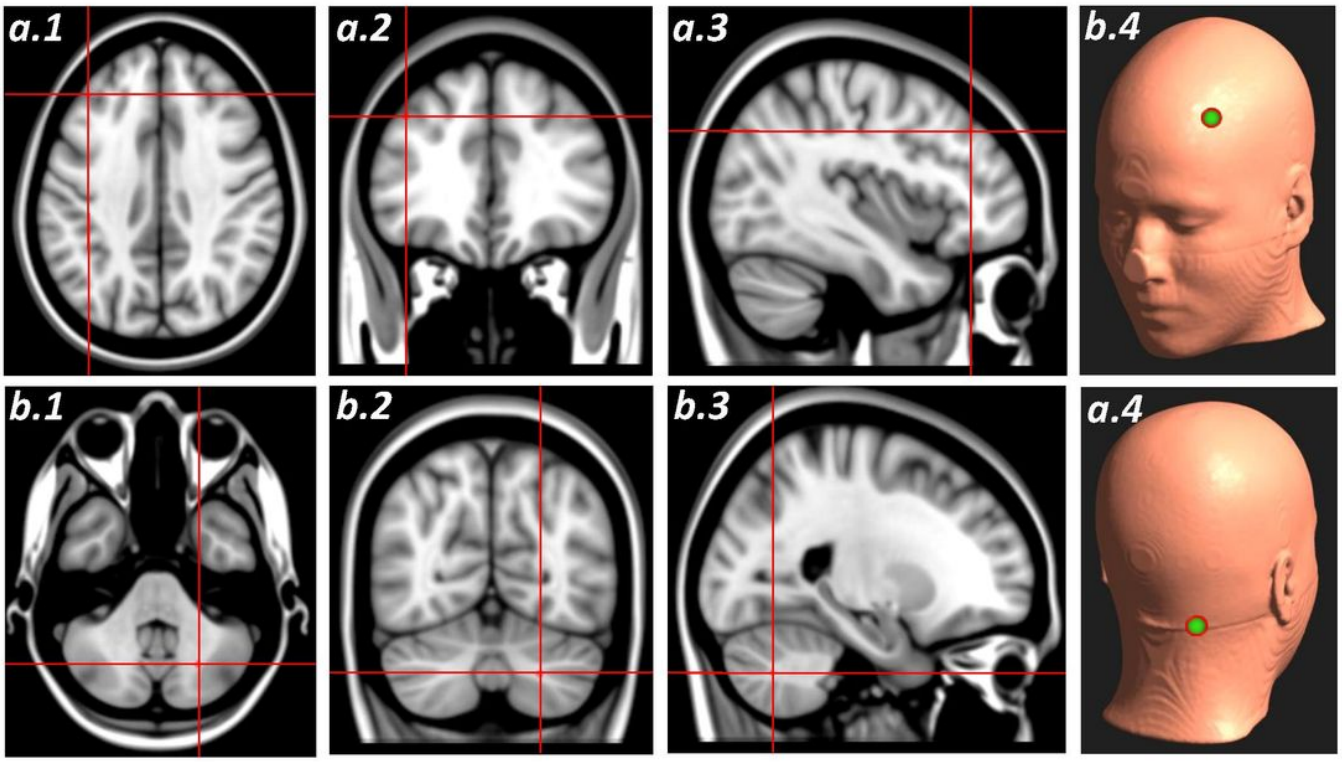

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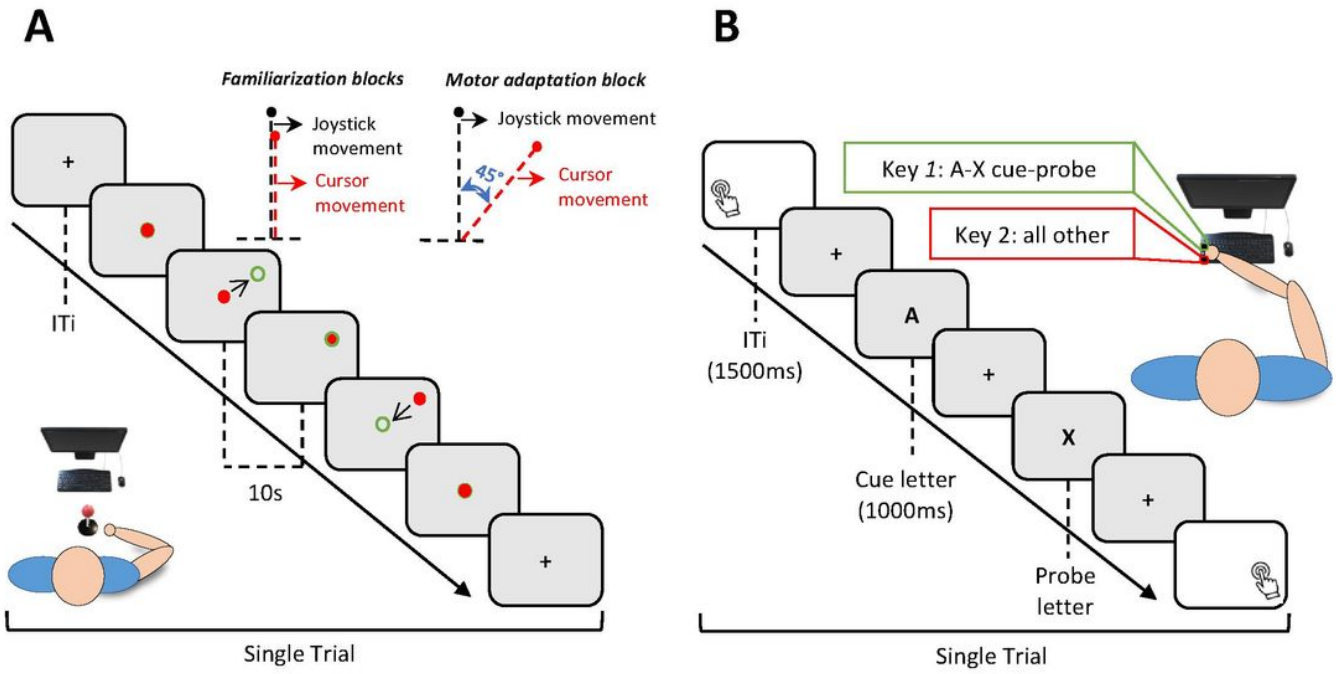

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