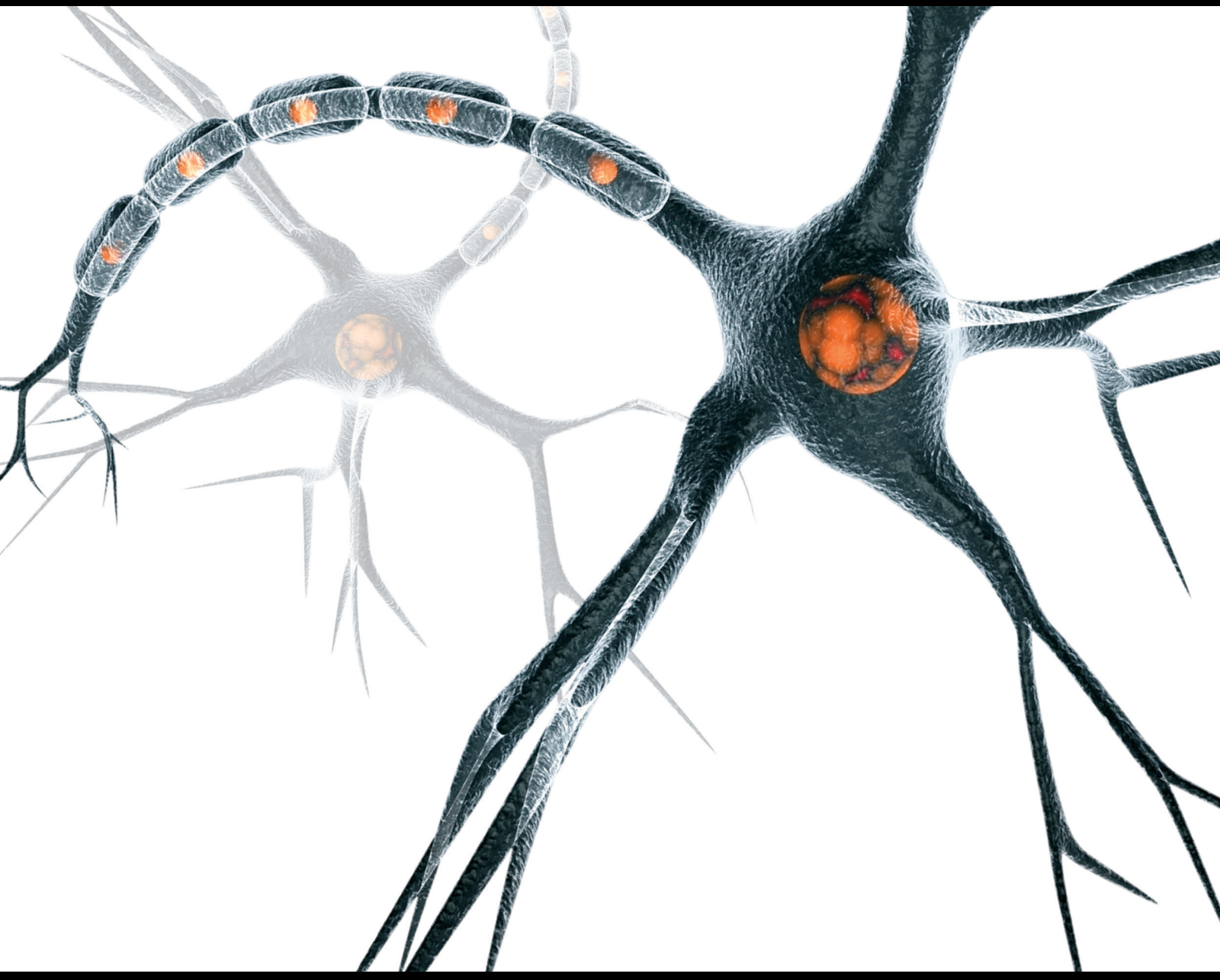
Neural Plasticity

Rehabilitation Induced Neural Plasticity in Diseases of the Central Nervous System 2020

Lead Guest Editor: Andrea Turolla
Guest Editors: Dario Farina, Vincent Chi Kwan Cheung, and Nick Ward



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Research Article

Contralesional Cathodal Transcranial Direct Current Stimulation Does Not Enhance Upper Limb Function in Subacute Stroke: A Pilot Randomized Clinical Trial

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Transcranial direct current stimulation (tDCS) has the potential to improve upper limb motor outcomes after stroke. According to the assumption of interhemispheric inhibition, excessive inhibition from the motor cortex of the unaffected hemisphere to the motor cortex of the affected hemisphere may worsen upper limb motor recovery after stroke. We evaluated the effects of active cathodal tDCS of the primary motor cortex of the unaffected hemisphere (ctDCSM1$_{UH}$) compared to sham, in subjects within 72 hours to 6 weeks post ischemic stroke. Cathodal tDCS was intended to inhibit the motor cortex of the unaffected hemisphere and hence decrease the inhibition from the unaffected to the affected hemisphere and enhance motor recovery. We hypothesized that motor recovery would be greater in the active than in the sham group. In addition, greater motor recovery in the active group might be associated with bigger improvements in measures in activity and participation in the active than in the sham group. We also explored, for the first time, changes in cognition and sleep after ctDCSM1$_{UH}$. Thirty subjects were randomized to six sessions of either active or sham ctDCSM1$_{UH}$ as add-on interventions to rehabilitation. The NIH Stroke Scale (NIHSS), Fugl-Meyer Assessment of Motor Recovery after Stroke (FMA), Barthel Index (BI), Stroke Impact Scale (SIS), and Montreal Cognitive Assessment (MoCA) were assessed before, after treatment, and three months later. In the intent-to-treat (ITT) analysis, there were significant GROUP*TIME interactions reflecting stronger gains in the sham group for scores in NIHSS, FMA, BI, MoCA, and four SIS domains. At three months post intervention, the sham group improved significantly compared to posttreatment in FMA, NIHSS, BI, and three SIS domains while no significant changes occurred in the active group. Also at three months, NIHSS improved significantly in the sham group and worsened significantly in the active group. FMA scores at baseline were higher in the active than in the sham group. After adjustment of analysis according to baseline scores, the between-group differences in FMA changes were no longer statistically significant. Finally, none of the between-group differences in changes in outcomes after treatment were considered clinically relevant. In conclusion, active CtDCSM1$_{UH}$ did not have beneficial effects, compared to sham. These results were consistent with other studies that applied comparable tDCS intensities/current densities or treated subjects with severe upper limb motor impairments during the first weeks post stroke. Dose-finding studies early after stroke are necessary before planning larger clinical trials.

1. Introduction

Stroke is a leading cause of disability worldwide. Hand paresis affects up to 80% of the subjects in the acute phase after ischemic stroke and substantially contributes to disability [1, 2]. Over the past several decades, transcranial direct current stimulation (tDCS) has emerged as a potential tool to enhance upper limb motor recovery [3–9].

The motor cortex of the unaffected hemisphere ($M1_{UH}$) may have a maladaptive role in motor recovery by overinhibition of the motor cortex of the affected hemisphere ($M1_{AH}$) according to the theory of interhemispheric inhibition [10]. Cathodal tDCS to inhibit $M1_{UH}$ ($ctDCSM1_{UH}$) and hence disinhibit $M1_{AH}$ has been investigated as a potential add-on therapy to upper limb rehabilitation. Until now, there is limited information about the effects of $ctDCSM1_{UH}$ during the first weeks after stroke when mechanisms of neuroplasticity are more active. Effective rehabilitation strategies delivered in this early phase are deemed pivotal to enhance recovery [11–17]. Meta-analyses concluded that $ctDCSM1_{UH}$ may be beneficial for improvement of upper limb function when delivered in the chronic phase, but not at earlier stages after stroke [8, 18]. However, most of the research included subjects in chronic than in early stages.

Only five studies focused on the effects of $ctDCSM1_{UH}$ in the subacute phase after stroke [12, 13, 15, 16]. In the acute phase, up to seven days after stroke according to the definition of the Stroke Recovery and Rehabilitation Roundtable taskforce [19], two studies assessed the effects of tDCS. In summary, the time of stroke onset varied from less than 10 days to less than 10 weeks; the numbers of treatment sessions were 2, 6, 9, 10, 15, or 30; treatment was administered on consecutive days in most studies except for one [16]; current intensities were 1, 1.5, or 2 mA with estimated current densities varying from 0.029 to 0.08 mA/cm^2. In regard to timing (before, during, or after other rehabilitation intervention), two out of seven trials delivered tDCS before therapy [12, 13], four during therapy [13, 15–17], and one did not include any therapy [20]. Rehabilitation interventions were very diverse, including physical therapy, occupational therapy, robot-aided therapy, or motor practice.

In addition to the paucity of data and the variety of paradigms in the few studies that addressed the effects of $ctDCS_{UH}$ in the subacute stage, a systematic review concluded that there is limited information about adverse events of tDCS in subjects post stroke [21].

The main objective of this study was to assess safety. Our primary findings, published elsewhere, showed that the active intervention was safe, compared to sham [22]. We also collected preliminary data regarding efficacy to inform plans for larger trials.

We hypothesized that motor recovery would be greater in the active than in the sham group. In addition, greater motor recovery in the active group might be associated with bigger improvements in measures in activity and participation in the active than in the sham group. Effects of $ctDCS_{UH}$ on cognition or sleep in stroke are largely unknown [23–25]. For this reason, we also assessed, for the first time, measures of cognition and sleep before and after treatment.

Here, we report the results of changes in the following secondary outcome measures of this pilot clinical trial: motor performance, spasticity, and use of the paretic upper limb in activities of daily living, as well as neurological impairment, disability, quality of life, sleep, and cognition.

2. Materials and Methods

2.1. Design. The study was a randomized parallel, two-arm, double-blind, sham-controlled clinical trial performed at the Albert Einstein Hospital from April, 2015, to September, 2017. The protocol was approved by the hospital's Ethics Committee and registered at clinicaltrials.gov (NCT 024555427). The research was conducted according to standards of the declaration of Helsinki and Brazilian regulations and with institutional guidelines. Informed consent was required from all participants and could be provided in writing by proxies for those unable to sign due to severe motor impairment. The independent Hospital Israelita Albert Einstein Institutional Review Board reviewed the clinical research and informed consent forms, every six months.

2.2. Participants. We included subjects in the acute (up to 7 days) or early subacute (from 7 days to 3 months) phases after stroke [19]. Inclusion criteria are as follows: age ≥ 18 years; ischemic stroke at least 72 hours and up to six weeks before enrollment, confirmed by CT or MRI; upper limb paresis defined as a minimum score of 1 in subitem 5a or 5b of the National Institutes of Health Stroke Scale (NIHSS) [26]; and ability to understand the protocol and provide informed consent.

Exclusion criteria are as follows: advanced systemic disease; clinical instability such as uncontrolled cardiac arrhythmia or heart failure; dementia; history of prior stroke affecting the corticospinal tract of $M1_{UH}$; strokes affecting the cerebellum or cerebellar pathways; contraindications to tDCS [27]; Modified Rankin Scale > 2 prior to stroke [26]; pregnancy; contraindication for physical therapy; and comprehension aphasia.

Demographic characteristics, history of hypertension, diabetes mellitus or prior stroke, handedness, performance of thrombolysis for ischemic strokes, time from stroke, and side, type, and etiology of stroke were registered in all subjects. Involvement of primary motor cortex and/or the posterior limb of the internal capsule in brain MRIs (fluid-attenuated inversion recovery images) performed on 3T scanners prior to treatment was also assessed by an experienced neuroradiologist, blinded to group assignment.

2.3. Experimental Protocol

2.3.1. Enrolment, Randomization, and Blinding. Recruitment was performed from our hospital admissions and from the community [28]. A computer-generated randomization schedule (10 blocks of 4 subjects) was created with *randomization.com* for allocation to either the active or sham $ctDCSM1_{UH}$ group at a 1 : 1 ratio. Subjects were consecutively enrolled in the study. For instance, if three patients had been included in the study, patient 4 was assigned the condition specified for the fourth included patient. Block

randomization assures that a determined proportion of subjects will be included in each group after a certain number of subjects have been included, keeping the proportions of participants in the active and sham groups as similar as possible to desired proportions throughout the study [29].

The randomization table was kept in a locked cabinet and in password-protected files, accessible only to the investigator who administered tDCS and the principal investigator.

Patients and researchers who administered physical therapy or evaluated outcomes were not aware of group assignment.

2.3.2. Intervention. Participants underwent three sessions of treatment per week over two weeks (total of six sessions) (Figure 1). In each session, a rubber sponge anode (7×5 cm) soaked in saline solution was placed over the ipsilesional supraorbital area and fixed by a nonconducting, nonabsorbent elastic strap. The cathode was placed on the contralesional C3/C4 position according to the EEG 10-20 reference system [27, 30]. The intensity of stimulation was 1 mA, and ramps up and down lasted for 10 seconds (DC-stimulator plus, Neuroconn, Germany).

In the active group, tDCS was applied for 20 minutes, and in the sham group, for 30 seconds including the ramping [30]. The supraorbital region was covered after active or sham ctDCSM1$_{UH}$. This sham setup reduces bias from unblinding [31]. Physical therapy was delivered after the end of stimulation with 30-minute exercises focused on the upper limb (details of the physical therapy interventions are provided as Supplementary Methods and Supplementary Table S1).

To date, there is no consensus or guidelines regarding the optimal intensity (i.e., 1 mA versus 2 mA), interval between sessions (i.e., every other day or consecutive sessions), duration (i.e., 15, 20 min, 30 min, or 40 min), or best timing to deliver physical therapy (concomitant with stimulation versus after the stimulation) [25, 32, 33]. We chose an intensity of 1 mA because higher intensities tend to provoke more paresthesias and could lead to unblinding [3]. We chose a total of 6 sessions with alternate days, in line with the average numbers of sessions (5-10) reported in the literature [12, 14–17]. TDCS was delivered before physical therapy, as performed by other studies that intended to prime cortical excitability prior to motor training [12, 14, 34]. A study about timing of tDCS and robot-aided therapy found that greater effects of tDCS in boosting the effects of training were obtained when tDCS was performed before, compared to during or after training [35].

2.3.3. Outcomes. The primary outcome of this study was safety, and the results were published elsewhere [22]. Secondary efficacy outcomes were assessed before the first session of treatment, after the last session of treatment, and three months later with the following behavioural measures: for upper limb motor impairment, the subitem 5a or 5b of the National Institutes of Health Stroke Scale (NIHSS$_5$) [26] and the Upper Limb Fugl-Meyer Assessment of Motor Recovery after Stroke (FMA, maximum motor function score = 66) [36]; for upper limb use in daily living, the Motor Activity Log (MAL) [37]; for upper limb spasticity, the Modified Ashworth Scale (MAS) [38]; for overall neurologic impairment, the National Institutes of Health Stroke Scale total score (NIHSS$_{total}$) [26]; for overall disability, the Modified Rankin Scale (mRS) [26]; for functional independence, the Barthel Index (BI) [26]; for quality of life, the Stroke Impact Scale (SIS) [39]; for cognition, the Montreal Cognitive Assessment (MoCA) [40]; and for sleep, the Pittsburgh Sleep Quality Index (PSQI) questionnaire [41]. Details of the secondary outcomes are provided in the Supplementary Material (Protocol Section: Clinical Outcomes).

2.3.4. Sample Size. Sample size was not formally determined based on prior data because the main goal of this study was to assess safety. Measures of efficacy were secondary outcomes. The results of this pilot study were expected to contribute to sample size estimation for future, larger trials. It has been estimated that, for a parallel, pilot clinical trial, at least 12 subjects should be included per group [42].

2.3.5. Statistical Analysis. Between-group differences in baseline characteristics were assessed with chi-square tests for categorical variables, and unpaired t-tests or Mann–Whitney tests for continuous variables according to data distribution.

Outcomes were analyzed with Generalized Estimating Equations (GEE) with factors time (preintervention, postintervention, and after 3 months) and group (active or sham). GEE is used to analyze correlated data, particularly when analysis of variance assumptions are not met [43]. Regarding this model, we used a marginal normal distribution and identity or logarithmic link function for continuous variables [44]. We assumed a Poisson distribution with an identity link function and a first-order autoregressive correlation matrix for discrete variables. Post hoc analyses were performed with Bonferroni's correction for multiple comparisons.

In addition, we evaluated Minimal Clinically Important Differences (MCID) of the following outcomes described for subjects in the early phase post stroke: FMA (9 points) [45], qualitative MAL (1 point) [46], NIHSS (3 points) [47], mRS (1 point) [48], and BI (20 points) [49].

Intention-to-treat (ITT) and per-protocol analyses were performed. Missing observations were imputed with the Last Observation Carried Forward (LOCF). A per-protocol analysis was performed on data from patients who completed at least five sessions of treatment and all sessions of evaluation of outcomes.

3. Results

3.1. Subjects. Supplementary Figure S1 shows the flow of subjects through the protocol. One subject in the active and one in the sham group dropped out before the assessment of outcomes at baseline. Two subjects in the active and one in the sham group dropped out before the first session of treatment. Eleven subjects completed the treatment in the active and 13 in the sham group. A total of 30 subjects were randomized to either active ctDCSM1UH ($n = 15$) or sham ($n = 15$). There were no significant between-group differences regarding the amount of out-of-protocol

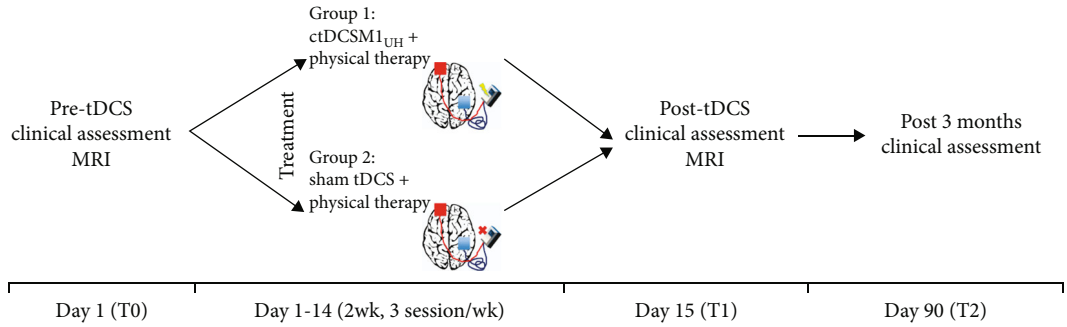

Figure 1: Experimental paradigm. ctDCSM1UH: cathodal transcranial direct stimulation of the motor cortex in the unaffected hemisphere.

physical therapy, during the intervention period or between the end of treatment and the 3-month follow-up (Supplementary Table S1). Information regarding amount of out-of-protocol physical therapy during the intervention period was not available for patients who dropped out. This occurred in only four patients in the active group and 2 patients in the sham group, out of 30 patients included in this study. Considering the period between the end of treatment and the 3-month follow-up, this information was not available for only 2 patients in the active group and 2 patients in the sham group out of 24 patients (Figure S1). Also, there were no between-group differences regarding the number of intervention sessions ($p = 0.355$). None of the subjects dropped out due to adverse events (for details, please see [22]).

Table 1 shows the subjects' characteristics. Lesions affecting the posterior limb of the internal capsule were significantly more frequent in the sham than in the active group. There were no significant differences between the groups at baseline, except for higher FMA scores in the active than in the sham group ($p < 0.001$) according to the GEE model in ITT and per-protocol analyses (Supplementary Tables S2-S4).

3.2. Outcomes: Pretreatment, Posttreatment, and Three Months Later. Tables 2 and 3 show the main *ITT* analyses of outcomes, except for SIS and PSQI scores (Supplementary Table S5). In ITT analysis, there were significant GROUP∗TIME interactions, reflecting the overall stronger gains in the sham group for scores in $NIHSS_{total}$, $NIHSS_5$, FMA, BI, MoCA, and four SIS domains ("activities of daily living," "hand function," "recovery," and "physical"). Interactions were not statistically significant for mRS, MAS, MAL, and PSQI scores or other SIS domains.

Immediately post treatment, both groups significantly improved compared to pretreatment in $NIHSS_{total}$, $NIHSS_5$, FMA, and BI scores, as well as in three SIS domains ("activities of daily living," "hand function," and "physical"). MoCA scores improved significantly in the sham group ($p < 0.001$) but not in the active group ($p > 0.99$). The active group improved significantly in the "recovery" SIS domain while no significant change was observed in the sham group.

At three months post intervention, the sham group improved significantly compared to posttreatment in FMA (Figure 2 and Supplementary Figure S2), $NIHSS_{total}$, BI, and three SIS domains ("activities of daily living," "physical," and "recovery) while no significant changes occurred in the active group. Also at three months, $NIHSS_5$ improved significantly in the sham group and worsened significantly in the active group (Figure 3 and Supplementary Figure S2). $NIHSS_5$ scores worsened from 0 pretreatment to 1 posttreatment in two subjects in the active, and in one subject in the sham group. Scores did not change in any other subject in the active and improved in two subjects in the sham group.

Supplementary Tables S3 and S4 show the main *per-protocol* analyses of outcomes, except for SIS and PSQI scores (Supplementary Tables S7-8). The per-protocol analysis showed similar results to ITT, except that there was no significant GROUP∗TIME interaction for $NIHSS_{total}$ and MoCA; at three months post treatment, both groups improved significantly in BI; also at three months, there were no significant changes in $NIHSS_5$ in either group.

Due to the imbalance in FMA scores (active > sham at baseline), we performed an additional GEE analysis of this outcome using the baseline FMA score as a covariate. The GROUP∗TIME interaction was no longer significant according to ITT and per-protocol analyses (Supplementary Tables S9-10). Therefore, according to ITT and per-protocol adjusted analyses, both sham and active groups improved significantly at 3 months compared to post treatment.

Table 3 shows MCID results according to the ITT analysis and Supplementary Table S11, to the per-protocol analysis. There were no significant GROUP∗TIME interactions for FMA, $NIHSS_{total}$, BI, MRS, or MAL according to either analysis.

4. Discussion

Overall, $ctDCSM1_{UH}$ was not beneficial, compared to sham, in any of the outcomes assessed in this study. There were no significant between-group differences in MCID for FMA, MAL, NIHSS, MRS, or BI. Also, there were no consistent between-group differences in spasticity, use of the paretic limb in activities of daily living, overall neurological impairments, cognition, or quality of sleep. Lower FMA scores in the sham group at baseline were consistent with a greater involvement of the PLIC in this group, compared to the active group. Between-group differences in FMA after

Table 1: Characteristics of the subjects.

Characteristic	Active tDCS ($n = 15$)	Sham tDCS ($n = 15$)	p value
Gender (female/male)	8/7	4/11	0.136[1]
Age, years (mean ± SD)	61.8 ± 15	61.9 ± 17.9	0.991[2]
Education, years (mean ± SD)	9.3 ± 4.1	7.5 ± 4.9	0.305[3]
Ethnicity, *n* (%)			0.478[4]
White	9 (60)	9 (60)	
Black	6 (40)	5 (33.3)	
Asian	0 (0)	1 (6.7)	
Hypertension, *n* (%)	10 (66.7)	12 (80)	0.682[5]
Diabetes mellitus, *n* (%)	7 (46.7)	6 (40)	0.713[1]
Right-handedness, *n* (%)	12 (85.7)	13 (92.9)	>0.999[5]
Previous stroke, *n* (%)	2 (13.3)	1 (7.1)	>0.999[5]
Time since stroke, median (IQR)	37 (23.5; 45.5)	26.5 (20.8; 37.3)	0.155[3]
Thrombolysis, *n* (%)	3 (20)	2 (13.3)	>0.999[5]
Lesion side (right/left/bilateral)	7; 8; 0	7; 7; 1	0.484[4]
HADS—depression, median (IQR)	3 (1; 6.5)	1.5 (0; 5.3)	0.246[3]
HADS—total score, median (IQR)	9 (4; 12)	4 (2; 11)	0.114[3]
Lesion site			
Corticosubcortical	9 (60)	5 (35.7)	0.191[1]
Subcortical	6 (40)	9 (64.3)	0.191[1]
Involved M1	6 (40)	4 (28.6)	0.700[5]
Involved PLIC	8 (53.3)	13 (92.9)	0.035[5]
Stroke etiology, TOAST			0.610[4]
Large-artery atherosclerosis	2 (13.4)	2 (13.3)	
Small-vessel occlusion	0 (0)	1 (6.7)	
Other determined etiology	2 (13.4)	1 (6.7)	
Undetermined etiology[a]	1 (6.7)	1 (6.7)	
Undetermined etiology[b]	10 (66.7)	10 (66.7)	

tDCS: transcranial direct current stimulation. HADS: Hospital Anxiety Depression Scale. SD: standard deviation. IQR: interquartile range. M1: primary motor cortex. PLIC: posterior limb of the internal capsule. TOAST: according to criteria from the Trial of Org 10172 in Acute Stroke Treatment. [1]Chi-square test. [2]Student's *t*-test. [3]Mann-Whitney's test. [4]Likelihood ratio test. [5]Fisher's exact test. [a]Complete investigation. [b]Incomplete investigation.

treatment favoured the sham group but were no longer statistically significant after adjustments for baseline scores.

The only outcome that improved significantly in the active but not in the sham group was the "recovery" domain of the SIS, according to both ITT and per-protocol analyses. The reason for this finding is unclear, given that no between-group differences were found in other SIS domains or in other outcomes that impact recovery.

On the other hand, performance in the MoCA test improved in the sham but not in the active group, immediately after the end of treatment, according to ITT analysis. The lack of improvement in the active group might reflect a negative effect of ctDCSM1$_{UH}$ on cognition, possibly by disturbing functional connectivity among brain areas other than M1[50, 51], though this speculation remains to be confirmed with imaging and neurophysiological studies. The MoCA test is a screening tool [52], and more comprehensive cognitive evaluations should be included in future protocols of ctDCS in stroke, considering the large knowledge gap in the field.

In opposition to the lack of consistent between-group differences immediately post treatment, at three months later, both ITT and per-protocol analyses showed greater improvements in the sham than in the active group in NIHSS$_{total}$, NIHSS$_5$, BI, and three SIS domains ("activities of daily living," "physical," and "recovery"). The "physical status" domain evaluates the strength, activity of daily life, mobility, and upper extremity performance. There were no significant between-group differences in any of these outcomes prior to treatment; therefore, these results could point to a detrimental effect of ctDCSM1$_{UH}$. However, the lack of significant differences in MCID for NIHSS$_{total}$ and BI [47] indicates that the better results obtained in the sham than in the active group, at 3 months post treatment, were not clinically relevant.

The number of individuals included per group in this study was greater than in other studies that included patients in the subacute phase, except for Hesse et al. that included 32 patients in each group [13]. Hesse et al. only included patients with severe motor impairments. In contrast, we

Table 2: Outcomes assessed before the first session of treatment (Pre), after the last session of treatment (Post), and three months later ($Post_{3m}$): intention-to-treat analysis, Generalized Estimating Equation model. Median and interquartile ranges are given.

Outcomes	Active			Sham			p values		
	Pre	Post	$Post_{3m}$	Pre	Post	$Post_{3m}$	Group	Time	Interaction
$NIHSS_{total}$	6 (3; 13)	3 (3; 11)	4 (3; 11)	5 (4; 10)	5 (3; 10)	4 (1; 8)	0.173	<0.001	<0.001
$NIHSS_5$	2 (1; 4)	1 (0; 4)	1 (1; 4)	2 (1; 4)	1 (1; 4)	1 (1; 4)	0.866	<0.001	<0.001
FMA	46 (8; 56.8)	51 (16.8; 61.5)	52 (16.8; 61.8)	22.5 (8.8; 43.5)	38.5 (20.5; 55.8)	43 (16.8; 57.3)	0.015	<0.001	<0.001
mRS	3 (2; 4)	3 (2; 4)	3 (2; 4)	4 (3; 4)	3 (3; 3)	3 (2; 3)	0.689	0.012	0.910
BI	80 (47.5; 95)	85 (57.5; 100)	92.5 (61.3; 100)	65 (47.5; 77.5)	77.5 (67.5; 90)	85 (75; 100)	0.654	<0.001	<0.001
$MAS_{shoulder}$	0 (0; 1)	0 (0; 0)	0 (0; 0)	0 (0; 1)	0 (0; 0)	0 (0; 0.5)	0.717	0.010	0.176
MAS_{elbow}	0 (0; 1.25)	0.5 (0; 1)	0.5 (0; 1.25)	1 (0; 2)	1 (0; 2)	1 (0; 2)	0.279	0.588	0.975
MAS_{wrist}	0.5 (0; 2.25)	0 (0; 1)	0 (0; 1.25)	1 (0.8; 2)	1 (0; 2)	2 (0; 2)	0.148	0.039	0.296
$MAS_{fingers}$	0.5 (0; 1.25)	0 (0; 1)	0 (0; 1)	1 (0; 1)	0 (0; 1)	1 (0; 1.3)	0.587	0.016	0.702
$MAL_{quantitative}$	1.05 (0; 1.97)	2.41 (0; 3.5)	2.25 (0; 3.89)	0.1 (0; 0.4)	0.6 (0; 1.9)	0.8 (0; 3.5)	0.261	0.087	0.211
$MAL_{qualitative}$	0.89 (0; 1.67)	2.16 (0; 3.58)	2.41 (0; 3.65)	0 (0; 0.2)	0.7 (0; 1.3)	0.9 (0; 3.1)	0.264	0.095	0.183
MoCA	18 (9; 24)	19 (10; 23)	21 (8; 24)	16 (8; 20)	20 (12; 23)	19 (13; 23)	0.728	<0.001	0.001

tDCS: transcranial direct current stimulation. $NIHSS_{total}$: National Institutes of Health Stroke Scale total score (0-42). $NIHSS_5$: National Institutes of Health Stroke Scale, motor score (0-5). FMA: Fugl-Meyer Assessment of Motor Recovery after Stroke, upper limb motor score. mRS: Modified Rankin Scale. BI: Barthel Index. MAS: Modified Ashworth Scale. $MAL_{qualitative}$: subscale qualitative of Motor Activity Log. $MAL_{quantitative}$: subscale quantitative of Motor Activity Log. MoCA: Montreal Cognitive Assessment.

Table 3: Minimal clinically important differences for secondary outcomes. Intention-to-treat analysis, Generalized Estimating Equations model with binomial distribution.

Outcome	Active		Sham		p value		
	Pre-post n (%)	Post-3m n (%)	Pre-post n (%)	Post-3m n (%)	Group	time	interaction
Fugl-Meyer Assessment	6 (42.9)	0 (0)	8 (57.1)	0 (0)	0.727	0.001	0.727
Motor activity log, qualitative	5 (35.7)	0 (0)	4 (28.6)	3 (21.4)	0.433	0.068	0.114
National Institutes of Health Stroke Scale	2 (13.3)	0 (0)	2 (13.3)	2 (13.3)	0.421	0.839	0.472
Modified Rankin Scale	5 (33.3)	4 (26.7)	7 (46.7)	4 (26.7)	0.576	0.328	0.647
Barthel Index	1 (7.1)	0 (0)	2 (14.3)	1 (7.1)	0.382	0.967	0.987

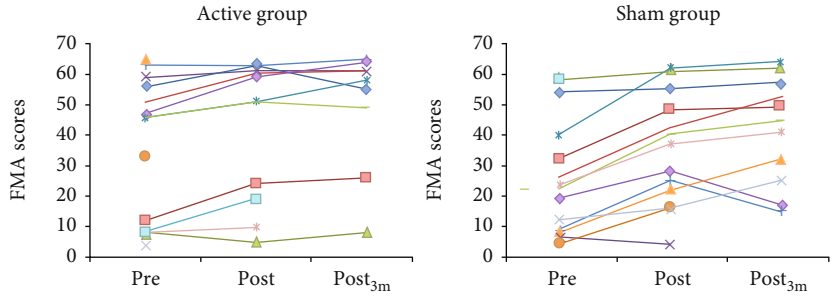

Figure 2: Absolute values of the Fugl-Meyer Assessment (FMA) of motor recovery after stroke scores at specific time points, for each participant in the active and sham groups.

included subjects with various levels of upper limb involvement. In addition, the chosen experimental paradigm (6 sessions delivered before physical therapy combined with ctDCSM1UH 1 mA intensity and estimated current density of 0.029 mA/cm^2) had not been previously reported in the subacute phase after stroke.

Despite these differences in the study design compared to prior research in the early phase after stroke, our results point to the same direction of all studies that chose stimulus intensities *below* 2 mA and estimated current densities *below* 0.057 mA/cm^2: we did not find significant between-group differences in outcomes related to upper limb impairment, function, overall neurologic impairment, or disability, immediately after the end of treatment. These results are consistent with a meta-analysis with substantial heterogeneity ($I^2 = 63.8$) [8], indicating that $ctDCSM1_{UH}$ does not lead to long-term enhancement of upper limb function when delivered at an early stage post stroke.

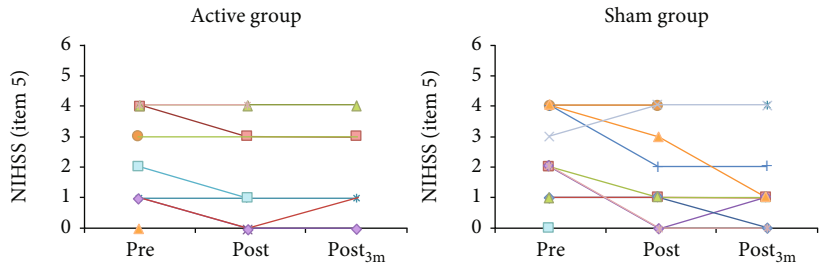

Figure 3: Absolute values of NIHSS (item 5a) scores at specific time points, for each participant in the Active and sham groups. NIHSS: National Institute of Health Stroke Scale.

Plasticity mechanisms are highly active during the first weeks after stroke. It is possible that ctDCSM1$_{UH}$ during this critical period does not have a positive impact on these mechanisms and the interhemispheric inhibition theory does not play an important role in many patients with stroke as previously argued [53–57]. Instead of being maladaptive, the activity of M1$_{UH}$ may be relevant to motor performance and recovery in these subjects, and hence, administration of ctDCSM1$_{UH}$ may be ineffective. The magnitude of endogenous plasticity may be higher than any effects of ctDCSM1$_{UH}$ during the first weeks and months.

On the other hand, Khedr et al. [14] (2 mA for 25 min, 6 sessions) found marginally positive results after treatment with ctDCS compared to sham in outcomes related to upper limb impairment. This could be related to paradigm choice: the timing of stimulation was performed in an earlier time window (17 days) than (around 4 weeks), and the current intensity was 2 mA. Only subjects who presented motor-evoked potentials in a hand muscle were included. Another study [15] also applied a 2 mA current intensity but reported positive results only after 6 months of follow-up in subjects with mild motor impairments. Estimated current densities were, respectively, 0.057 and 0.08 mA/cm^2. Conversely, Hesse et al. [13] chose a stimulus intensity of 2 mA (current density, 0.057 mA/cm^2) and did not report significant differences between active and sham groups but only included subjects with severe motor deficits.

The lack of significant effects of cTDCS in the early phase after stroke [58] may reflect the mechanisms of recovery, but it is also possible that paradigms of stimulation and eligibility criteria may explain discrepancies in results. A metaregression estimated that, overall, higher tDCS current densities may lead to greater motor recovery [59]. In addition, in patients in the chronic phase after stroke, an intensity of 4 mA was found to be safe [60], but until now, no studies evaluated intensities greater than 2 mA in earlier stages.

Overall, these results, together with our observations, provide key information for the design of future studies aiming at efficacy on motor outcomes: administration of ctDCSM1$_{UH}$ at stimulus intensities of at least 2 mA, current densities greater than 0.057 mA/cm^2 in patients with residual upper function, without severe deficits, may be associated with a greater likelihood of success. Tailoring the type of tDCS (cathodal or anodal) to each individual according to the severity of their deficits and/or phase after stroke is more likely to lead to benefit than applying these treatments to very different groups of subjects with stroke, a very heterogeneous condition. Clinical, neuroimaging, and neurophysiologic tools are expected to provide information about the underlying mechanisms of recovery that will allow the selection of the right patient to the right intervention at the right dose [61].

These conclusions cannot be extrapolated to other neuromodulation interventions such as rTMS [62, 63] or anodal tDCS administered during the first days and weeks after stroke. For instance, Andrade et al. [11] reported that functional independence assessed with the Barthel Index improved significantly more after anodal tDCS of the premotor or primary motor cortex in the affected hemisphere than after sham stimulation, in subjects at 1-3 months after stroke. In the three groups, tDCS was followed by constraint-induced movement therapy (CIMT) in 10 sessions of treatment. Subjects were recruited at a later subacute stage compared to those included in the present study. Differences in treatment schedule, time after stroke, type and target of tDCS, outcome (functional independence), and add-on rehabilitation paradigm (CIMT) may also explain discrepancies between these results and the findings of the present study.

These results should be viewed with caution considering the limitations of this study. First, behavioral measures were secondary outcomes and were collected in a relatively small sample of patients with a main goal of assessing the estimate of effect that would allow a formal sample size calculation for a further larger study. Second, we did not conduct a stratified randomization according to the level of impairment, and there was an imbalance in FMA scores at baseline. However, we also analyzed the data considering baseline FMA as a covariate, and there were no between-group differences. Third, biomarkers such as cortical excitability or severity of motor impairment were not part of the eligibility criteria. Until now, there is no consensus about evidence-based biomarkers that should be used in trials of neuromodulation in stroke. There is a deep need for clinical, imaging, or other variables that can help tailor treatments [64]. Another potential limitation is that the principal investigator, and not an independent investigator, sent the information about the computer-generated randomization schedule to the researcher who administered tDCS. Finally, the duration of upper limb therapy may have been insufficient. There is still no consensus about the best duration of upper limb therapy combined with tDCS in the subacute stage after stroke. The VECTORS study showed an absence of benefit of an

intensive intervention of motor training, constraint-induced movement therapy, compared to usual care in the subacute phase after stroke [65], in the absence of add-on neuromodulation interventions. This finding contrasts with results of CIMT in patients in the late subacute and chronic stages after stroke [66]. Yet, it is possible that tDCS may be beneficial when longer durations of training are provided, compared to those administered in our study. It is also possible that fatigue in subacute patients or maladaptive effects of "excessive" stimulation with prolonged training limit the potential benefits of longer therapy sessions. Future studies are necessary to define not only the dose of $CtDCSM1_{UH}$ but also the optimal "dose" of therapy applied in combination with $CtDCSM1_{UH}$.

5. Conclusion

In summary, our data provide evidence that $CtDCSM1_{UH}$ in the early phase after stroke did not have consistent beneficial effects on motor impairments, disability, or quality of life, immediately after treatment or three months later. Early phase dose-finding studies after stroke are necessary before planning larger clinical trials.

Data Availability

The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

DSB and ABC collected the data, performed stimulation therapy, analyzed and interpreted the data, and prepared the text of the manuscript. JP prepared Figure 2. DS, PS, PFF, and DSS performed physical therapy. PL and SL performed cognitive evaluation. All authors reviewed the manuscript.

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Supplementary Materials

Figure S1. Flow of subjects throughout the study, Figure S2. Changes in Fugl-Meyer Assessment of Motor Recovery after Stroke (FMA) and NIH Stroke Scale (item 5) scores at specific time points, according to intention to treat (ITT, left) and per protocol (right) analyses. Error bars represent the standard error for 15 patients in each group (n= 30, ITT, left), 9 patients in the active group and 11 patients in the sham group (n=20, per protocol, right). ∗ Statistically significant differences. Table S1. Hours of physical therapy out of protocol as reported by the patients, between the first and last session of transcranial direct current stimulation (tDCS) and between the last session and three months later. Table S2. Post-hoc intention-to-treat analyses of: motor subitem (5a) of the National Institutes of Health Stroke Scale, Fugl-Meyer Assessment of Motor Recovery after Stroke -Upper limb motor score, Modified Ashworth Scale, Barthel Index, National Institutes of Health Stroke Score (total scale) and Modified Rankin Scale. Table S3. Outcomes assessed before the first session of treatment (Pre), after the last session of treatment (Post) and three months later ($Post_{3m}$): per-protocol analysis, Generalized Estimating Equation model. Median and interquartile ranges are given. Table S4. Post-hoc per-protocol analyses of: Motor subitem (5a) of the National Institutes of Health Stroke Scale, Fugl-Meyer Assessment of Motor Recovery after Stroke - Upper limb motor score, Modified Ashworth Scale, Barthel Index, Motor Activity Log. Table S5. Stroke Impact Scale (SIS) and Pittsburgh Sleep Quality Index assessed before the first session of treatment (Pre), after the last session of treatment (Post) and three months later ($Post_{3m}$): intent-to-treat analysis, Generalized Estimating Equation model. Median and interquartile ranges are given. Table S6. Stroke Impact Scale (SIS). Post-hoc, intention-to-treat analysis. Table S7. Stroke Impact Scale (SIS) and Pittsburgh Sleep Quality Index assessed before the first session of treatment (Pre), after the last session of treatment (Post) and three months later ($Post_{3m}$): per-protocol analysis, Generalized Estimating Equation model. Median and interquartile ranges are given. Table S8. Stroke Impact Scale (SIS). Post-hoc, per-protocol analysis. Table S9. Generalized Estimating Equations, intention-to-treat (ITT) and per-protocol analyses of Fugl-Meyer Assessment (FMA) scores, adjusted for pre-treatment FMA scores (covariate). Table S10. Post-hoc intention-to-treat and per-protocol analyses of Fugl-Meyer Assessment (FMA) scores, adjusted for baseline FMA scores (covariate). Table S11. Minimal clinically important difference. Per-protocol analysis of changes before and after treatment (Pre-Post), as well as post-treatment compared to 3 months after treatment (Post-$Post_{3m}$). *(Supplementary Materials)*

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Research Article

Neural Correlates of Motor Recovery after Robot-Assisted Training in Chronic Stroke: A Multimodal Neuroimaging Study

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Stroke is a leading cause of motor disability worldwide, and robot-assisted therapies have been increasingly applied to facilitate the recovery process. However, the underlying mechanism and induced neuroplasticity change remain partially understood, and few studies have investigated this from a multimodality neuroimaging perspective. The current study adopted BCI-guided robot hand therapy as the training intervention and combined multiple neuroimaging modalities to comprehensively understand the potential association between motor function alteration and various neural correlates. We adopted EEG-informed fMRI technique to understand the functional regions sensitive to training intervention. Additionally, correlation analysis among training effects, nonlinear property change quantified by fractal dimension (FD), and integrity of M1-M1 (M1: primary motor cortex) anatomical connection were performed. EEG-informed fMRI analysis indicated that for iM1 (iM1: ipsilesional M1) regressors, regions with significantly increased partial correlation were mainly located in contralesional parietal, prefrontal, and sensorimotor areas and regions with significantly decreased partial correlation were mainly observed in the ipsilesional supramarginal gyrus and superior temporal gyrus. Pearson's correlations revealed that the interhemispheric asymmetry change significantly correlated with the training effect as well as the integrity of M1-M1 anatomical connection. In summary, our study suggested that multiple functional brain regions not limited to motor areas were involved during the recovery process from multimodality perspective. The correlation analyses suggested the essential role of interhemispheric interaction in motor rehabilitation. Besides, the underlying structural substrate of the bilateral M1-M1 connection might relate to the interhemispheric change. This study might give some insights in understanding the neuroplasticity induced by the integrated BCI-guided robot hand training intervention and further facilitate the design of therapies for chronic stroke patients.

1. Introduction

Stroke is the leading cause of death worldwide, and the survivors undergo various disabilities related to motor, sensory, and cognitive functions. Specifically, robot-assisted therapy is a kind of task-specific and high-intensity exercise in an active, functional, and highly repetitive manner [1]. It has been proven to be efficient to induce neuroplasticity modulation and promising long-term motor recovery [2]. A brain computer interface (BCI) can facilitate stroke rehabilitation by integrating the exoskeleton robots to develop the BCI-guided robot-assisted therapy, which is believed to engage various brain functional regions [3] in the recovery process.

Electroencephalography (EEG), which can capture subtle neurological changes, has been widely used in studying neural functions. EEG signals result from the mixture of propagating electric potential fluctuations, mainly reflecting the postsynaptic activity of large populations of cortical pyramidal cells [4]. Additionally, functional magnetic resonance imaging (fMRI) has become one of the most commonly used neuroimaging tools to assess the cortical alterations associated with learning, diseases, or rehabilitation [5]. Resting-state fMRI that measures the temporal correlation of the blood oxygen level-dependent (BOLD) signal between different regions at resting state has emerged as a powerful tool to map the functional organization of the brain [6]. fMRI measurements have

an excellent spatial resolution in millimeters but low temporal resolution limited to few seconds. While EEG holds millisecond-level temporal resolution, allowing the adequate sampling of the rapidly changing electrical dynamics of neuronal populations [4]. Since EEG and fMRI exhibit highly complementary characteristics, their integration has been widely exploited [7, 8]. Simultaneously recorded EEG and fMRI data make it possible to integrate these two neuroimaging modalities and have received substantial attention [9]. In our current study, we also adopted a concurrent EEG-fMRI technique to figure out related functional regions sensitive to the training effect. It should be noted that researchers have put numerous efforts to detecting these significant functional regions based on various neuroimaging techniques. For example, some studies have indicated the crucial role of supplementary motor area (SMA) in a motor system to execute various tasks including interlimb coordination [10] and many unimanual tasks involving movement sequencing as well as internal pacing [11]. Specifically, for stroke patients, the reduced partial correlation between SMA and M1 together with the interhemispheric correlation of both SMAs during visually paced hand movements has been found [12]. The reduced partial correlation between ipsilesional of SMA and M1 was also exhibited when stroke patients perform hand movements [13] and index finger-tapping task [14]. Meanwhile, it is noted that improved motor function of stroke patients might be highly correlated to a restitution of ipsilesional effective connectivity between SMA and M1 [15] and functional connectivity of the ipsilesional M1 with contralesional SMA [16]. Hence, in our study, we hypothesized SMA would also play an essential role in motor recovery with BCI-guided robot-hand training.

Quantification of EEG signal can be linked to the clinical features, such as the rehabilitation progress in chronic stroke. Nonetheless, due to the volume-conduction effect, the activities of scalp EEG signal are often assumed to come from multiple sources spatially dispersed in the brain cortex, which blurs the underlying neural mechanisms [17]. Therefore, EEG source imaging has emerged where the patient-specific anatomical properties could be taken into account using individual structural MRI images to disentangle useful neural information. However, few studies leveraged the indicators derived from EEG source space to investigate motor training effects for chronic stroke patients.

Although linear measurements have been widely recognized to reflect the brain characteristics, there is a growing tendency that different nonlinear measures have been proposed to depict the complexity of EEG signals and adopted to predict treatment response to repetitive transcranial magnetic stimulation in depression [18], evaluate the effect of stroke rehabilitation [19], and facilitate the classification system for hand recovery in stroke patients [20]. Fractal property that is quantified by the fractal dimension (FD) [21] is a nonlinear descriptor for brain signals, including EEG signals, which is closely associated with specialization and efficiency of brain functioning [22]. Investigation of such fractal nature as its correlation with the rehabilitation process for patients with neurological disorders, including stroke, is particularly important. The interhemispheric imbalance, especially the imbalance between homologous primary motor regions, always plays a crucial role in stroke motor rehabilitation [23]. Additionally, structural imaging, such as diffusion tensor imaging (DTI), has provided pivotal insights into the functional role of the underlying structural tracts in stroke-related changes [24]. Reductions in fractional anisotropy (FA), a DTI-derived measure of degree of anisotropy in white matter (WM), have been found in stroke individuals [25]. Specifically, the integrity of transcallosal motor fibers may play a role in monitoring the treatment response in chronic stroke [26].

The purpose of this study is to investigate the neural correlates of motor recovery after BCI-guided robot-assisted training in chronic stroke from a multimodality neuroimaging perspective. The EEG-informed fMRI analysis was utilized to locate the potential functional brain regions sensitive to the training effect. Furthermore, we hypothesized that the training effect should be related to the interhemispheric interaction change and such induced change was supposed to be based on the structural substrates connecting bilateral primary motor areas. Hence, the corresponding correlation analyses were performed to verify these hypotheses.

2. Materials and Methods

2.1. Subjects. Fourteen chronic stroke subjects (13 males, mean age = 54 ± 8 years, right-handedness) with unilateral hemispheric impairment were recruited from local community. The inclusion criteria were as follows: (1) first-ever stroke, (2) more than 6 months since the stroke onset prior to the experiment, (3) a single unilateral brain lesion, (4) Hong Kong Montreal Cognitive Assessment (HK-MoCA) [27, 28] score ≥ 22 to ensure sufficient cognitive function to understand instructions and perform tasks, (5) moderate to severe paretic hand dysfunctions (Fugl-Meyer Assessment score for upper extremity < 47), and (6) no additional rehabilitation therapies applied to the patient. The exclusion criteria were as follows: (1) aphasia, neglect, apraxia, history of alcohol, drug abuse, or epilepsy; (2) severe hand spasticity; (3) hand deformity and wound; and (4) severe cognitive deficits. Motor functions of the paretic upper limbs for all stroke subjects were assessed with Fugl-Meyer Assessment for upper extremity (FMA) which is a reliable and widely used measurement [29] before and after the intervention, respectively. Table 1 summarizes the demographics and clinical properties of subjects.

2.2. Training Interventional Protocol. All subjects received a 20-session BCI-guided robot hand training therapy with an intensity of 3-5 sessions per week and completed the whole training process with 5-7 weeks. During each training session, the surface EEG signals of each subject were acquired using 16 electrodes (C1, C2, C3, C4, C5, C6, Cz, FC1, FC2, FC3, FC4, FCz, CP1, CP2, CP3, and CP4 according to international 10-20 system) at a sample rate of 256 Hz. The EEG signals were then amplified (g.LADYbird, g.USBamp, g.Tec Medical Engineering GmbH, Austria) and processed to generate the real-time topography of the brain electrical potential for surveillance. A paradigm with a fixed sequence

Table 1: Demographics and clinical properties of the participants.

No.	Age range	Gender	Stroke onset (years)	Lesion side	Lesion location	Stroke type	FMA (Max score: 66)	
							Pre	Post
S1	45-49	M	1	R	MFG, SFG, precentral, supramarginal, SMA	Ischemic	19	34
S2	65-69	M	8	L	Insula, putamen, IFG, temporal pole	Hemorrhage	22	27
S3	65-69	M	1	R	Insula, ITG, IOG, putamen	Hemorrhage	13	16
S4	60-64	M	3	R	Insula, putamen, IFG Rolandic operculum	Ischemic	16	14
S5	45-49	M	0.7	R	ITG, MTG, STG, MOG, angular, supramarginal	Hemorrhage	17	25
S6	60-64	M	11	L	PLIC, putamen, insula, postcentral, SFG	Ischemic	22	24
S7	55-59	M	6	R	Insula, IFG Rolandic operculum	Ischemic	13	23
S8	40-44	M	5	R	Insula, Rolandic operculum, IFG, STG, putamen, temporal pole	Hemorrhage	15	17
S9	50-54	F	3	L	Insula, Rolandic operculum, putamen	Hemorrhage	34	34
S10	40-44	M	3	R	Insula, MTG, STG, temporal pole, putamen, Rolandic operculum	Hemorrhage	17	20
S11	55-59	M	5	L	Insula, IFG, putamen	Hemorrhage	28	33
S12	50-54	M	1	L	Putamen, caudate nucleus	Ischemic	24	22
S13	55-59	M	7	R	Putamen, temporal pole, IFG, insula, Rolandic operculum	Ischemic	20	25
S14	45-49	M	1	R	Insula, putamen	Hemorrhage	34	37

F: female; FMA: Fugl-Meyer Assessment for upper limb; IFG: inferior frontal gyrus; IOG: inferior occipital gyrus; ITG: inferior temporal gyrus; L: left hemisphere lesion; M: male; MFG: middle frontal gyrus; MOG: middle occipital gyrus; MTG: middle temporal gyrus; PLIC: posterior limb of the internal capsule; SFG: superior frontal gyrus; SMA: supplementary motor area; STG: superior temporal gyrus; R: right hemisphere lesion.

showing instructions for motor imagery was played, during which the subjects were guided to imagine either grasping or releasing a cup following commands. EEG signal from C3 or C4 channel according to the subject's lesion side was extracted to calculate the α suppression [30]. The EEG data were transformed into the frequency domain using Fourier transform, and the mean power in the α band (8-13 Hz) was derived. Then, the α suppression was calculated as follows:

$$\alpha \text{ suppression} = \frac{P_{\text{rest}} - P_{\text{MI}}}{P_{\text{rest}}}, \quad (1)$$

where P_{MI} and P_{rest} stand for the calculated α power during the motor imagery period and the resting-state, respectively. A trigger would be sent to an exoskeleton robot hand [31] (illustrated in Figure S1 B; a detailed description is provided in supplementary materials) to provide mechanical force and assist the paretic hand in grasping and opening if the α suppression exceeded 20% based on the previous study [32]. The success rate was defined as the percentage of correctly detected trials during motor imagery tasks at each session.

This study was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee. All subjects gave written consent before the intervention and underwent the experiments in the Chinese University of Hong Kong rehabilitation labs. This study was registered at https://clinicaltrials.gov (NCT02323061).

2.3. Data Acquisition. MRI scans were acquired for all the 14 subjects before and after the training sessions. A 3T Philips MR scanner (Achieva TX, Philips Medical System, Best, Netherlands) with an 8-channel head coil was used to acquire high-resolution T1-weighted anatomical images (TR/TE = 7.47/3.45 ms, flip angle = 8°, 308 slices, voxel size = $0.6 \times 1.042 \times 1.042\text{mm}^3$) using a T1-TFE sequence (ultrafast spoiled gradient echo pulse sequence), and BOLD fMRI images (TR/TE = 2000/30 ms, flip angle = 70°, 37 slices/volume, voxel size = $2.8 \times 2.8 \times 3.5\text{mm}^3$) using an EPI sequence (gradient-echo echo-planar-imaging sequence). Besides, diffusion-weighted images were acquired using a diffusion-weighted single-shot spin-echo echo-planar pulse (DWISE) sequence (TR/TE = 3788/88 ms, flip angle = 90° from 32 directions, 60 slices/volume, voxel size = $1.5 \times 1.5 \times 2\text{mm}^3$). When acquiring resting-state fMRI data, subjects were presented with a white cross in a black background and instructed to rest while focusing on the fixation cross. The resting-state fMRI acquisition lasted for 8 minutes.

The EEG data were acquired simultaneously with the fMRI using Neuroscan system (SynAmps2, Neuroscan Inc., Herndon, USA). A 64-channel MR-compatible EEG cap according to a standard 10-20 system was utilized, combined with 2 extra electrocardiogram (ECG) electrodes attached at the left lower and near-midline upper chest and 1 electrooculogram (EOG) electrode placed below the right eye. All recording impedances were kept below 5 k. The reference channel was located at the point between Cz and CPz; an AFz electrode was treated as the ground. Signals were filtered between 0.1 and 256 Hz using an analog filter and sampled at 1000 Hz for off-line processing. The whole scheme of experimental protocol is shown in Figure S1 A.

2.4. Data Analysis. In our study, the analysis was mainly performed from multimodality perspective including fMRI, EEG, and DTI neuroimaging data, and the whole analysis pipeline is summarized in Figure 1. The left column included the preprocessing of DTI data, M1-M1 fiber tractography, and calculation of FA value of M1-M1 tract (please refer to section 2.7 in supplementary materials). The middle column included the analysis of EEG data including preprocessing, distributed source estimation, time series extraction from cM1 and iM1 seeds, and the calculation of indices characterizing nonlinear properties (please refer to sections 2.2, 2.3, and 2.6 in supplementary materials). The right column mainly included the preprocessing of fMRI data, iM1 EEG regressor construction, and integrated EEG-informed fMRI analysis (please refer to sections 2.1 and 2.4 in supplementary materials). The detailed description of each step is provided in the supplementary materials.

2.5. Statistical Analysis. Statistical analyses were performed using SPSS 25.0 (IBM SPSS Statistics, NY, US) with the significance level set at $p < 0.05$. A paired t-test between pretraining and posttraining was applied to examine if FMA score has changed after the intervention. For the conventional fMRI analysis, the paired t-test was performed for pre- and posttraining based on the individual partial correlation maps from 14 subjects. For EEG-informed fMRI analysis, two-way repeated-measure analysis of variance (ANOVA) was conducted with two fixed effects of time (pre- and posttraining) and frequency bands (theta, alpha, and beta) and with subject effect considered as a random effect for iM1 regressor. Paired t-tests as the post hoc analysis were further performed between pre- and posttraining based on the individual partial correlation maps for each frequency band. Multiple comparisons were corrected using Gaussian random field theory at the cluster level (voxel-wise significance: $p < 0.005$; cluster-wise significance: $p < 0.05$) [33]. For the survived clusters, false discovery rate (FDR) correction was further performed ($p < 0.05$) [34]. Pearson's correlation coefficients were calculated between FMA score changes and interhemispheric asymmetry change before and after the training. To investigate the potential underlying structural base influencing the interhemispheric property alternation, correlation analysis was also conducted between interhemispheric asymmetry change and FA of M1-M1 anatomical connection.

3. Result

We first assessed the effect of training on motor functions in the stroke participants. A paired t-test indicated a statistically significant improvement in FMA scores following training intervention, from 21 ± 6.7 to 25 ± 7 ($t(13) = 3.313$, $p = 0.006$). Besides, an increasing trend of success rate along with 20 training sessions was observed, with the mean of 73.01% for the first five sessions to 76.78% for the last five sessions.

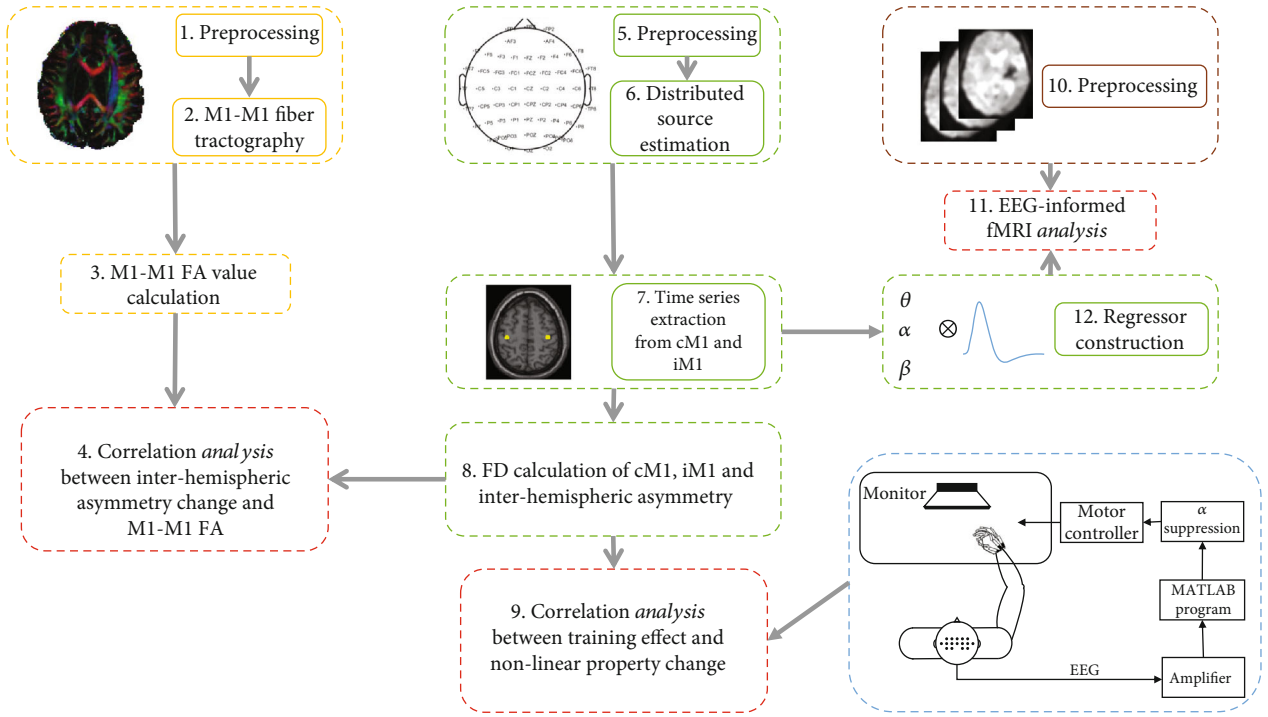


Figure 1: Illustration of analysis pipeline. The whole analysis included processing of fMRI, EEG, and DTI data; EEG-informed fMRI analysis; correlation analysis between training effect and nonlinear property change characterized by FD; and correlation analysis between interhemispheric asymmetry change and integrity of M1-M1 anatomical connection quantified by FA.

The two-way ANOVA indicated that clusters with significant time × frequency interaction were found in a cluster at bilateral SMA, paracentral lobule and contralesional superior frontal gyrus (BA6 C&I), a cluster at ipsilesional precentral and postcentral gyrus (BA4 I and BA6 I), and a cluster at contralesional superior parietal lobe (SPL) and inferior parietal lobe (IPL) (BA7 C) (Illustrated in Figure 2 and summarized in Table 2).

Paired t-tests were performed between pre- and post-training with different combinations of three representative EEG bands (theta, alpha, and beta). When theta band EEG signal was used as the regressor, significantly increased partial correlation was found in one cluster overlying the contralesional superior parietal gyrus, inferior parietal gyrus, and precuneus (BA7 C). Significantly decreased partial correlations were found in the ipsilesional precentral gyrus (BA4 I) and ipsilesional supramarginal gyrus (BA48 I). When alpha band EEG signal was used as the regressor, significantly increased partial correlations were found in a cluster involving the contralesional superior frontal gyrus and middle frontal gyrus (BA8 C and BA6 C) and the other clusters including the contralesional precuneus, cuneus, and superior occipital gyrus (BA7 C, BA19 C, and BA18 C). Significantly decreased partial correlation was found in the ipsilesional superior temporal gyrus (BA48 I). When beta band EEG signal was used as the regressor, significantly increased partial correlations were found in the contralesional postcentral gyrus (BA4 C) as well as a cluster covering contralesional SMA and superior frontal gyrus (BA6 C). Significantly decreased partial correlation was found in the ipsilesional superior temporal gyrus (BA48 I) (illustrated in Figure 3 and summarized in Table 3).

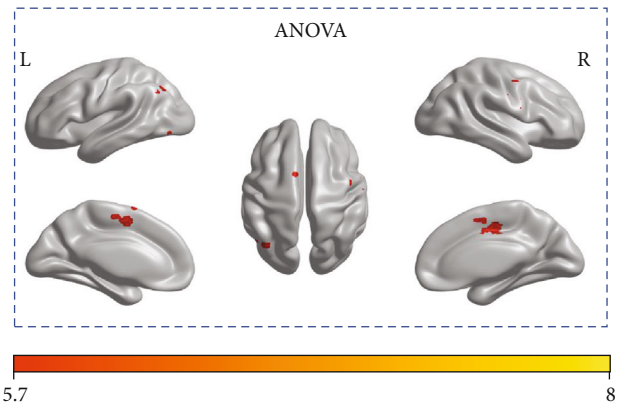


Figure 2: Surface rendering of brain functional regions which showed significant time × frequency interaction for iM1 regressor. The right side is the ipsilesional side.

For conventional seed-based fMRI connectivity analysis, paired t-test showed that significant clusters were observed mainly in contralesional Brodmann area 6 when seed ROI was located at iM1 (illustrated in Figure 4).

Then, we explored the relationship between training effect and nonlinear property changes quantified by FD. Pearson's correlation revealed that the FMA score change significantly correlated with interhemispheric asymmetry change (Figure 5(a), $r = -0.6219$, $p = 0.0352$; Bonferroni corrected) before and after training. We hypothesized that

Table 2: Brain regions showing significant time × frequency interaction.

C/I	Anatomical region	Peak MNI coordinate (x, y, z)		
iM1 regressor				
C&I	Supplementary motor area	-5	1	48
C&I	Paracentral lobule			
C	Superior frontal gyrus			
I	Precentral gyrus	60	8	26
I	Postcentral gyrus			
C	Superior parietal lobe	60	8	26
C	Inferior parietal lobe			

C/I: contralesional or ipsilesional.

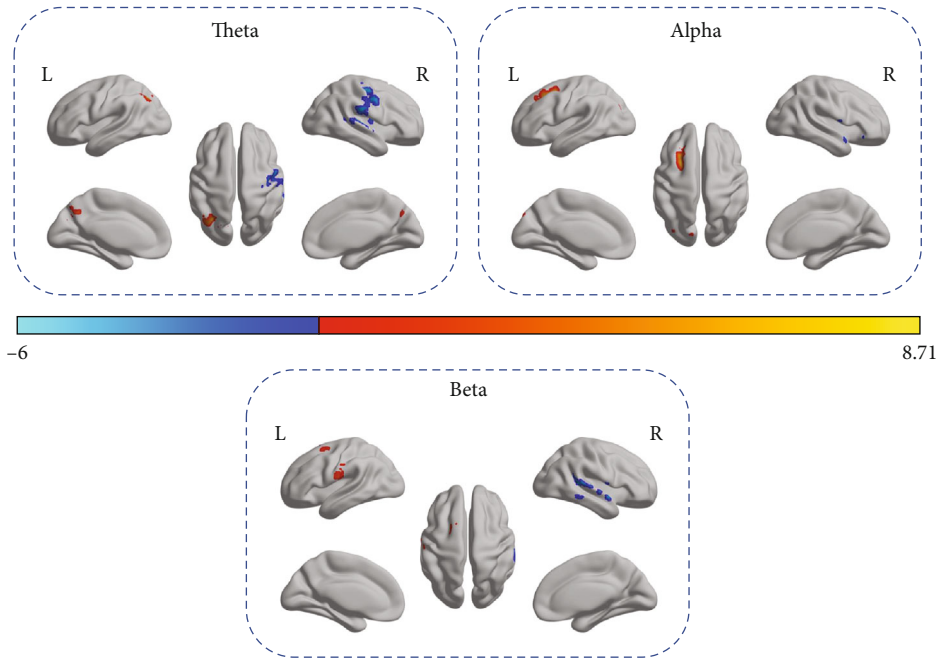


Figure 3: Surface rendering of brain functional regions which showed significant partial correlation change before and after training, given the regressor embedding spectral information (including theta, alpha, and beta frequency bands) derived from EEG source time course of iM1. The right side is the ipsilesional side.

the alteration of interhemispheric property would be associated with corresponding structural characteristics. Indeed, a significant relevance between interhemispheric asymmetry change and FA of M1-M1 anatomical connection was observed (Figure 5(b), $r = 0.6529$, $p = 0.0228$; Bonferroni corrected).

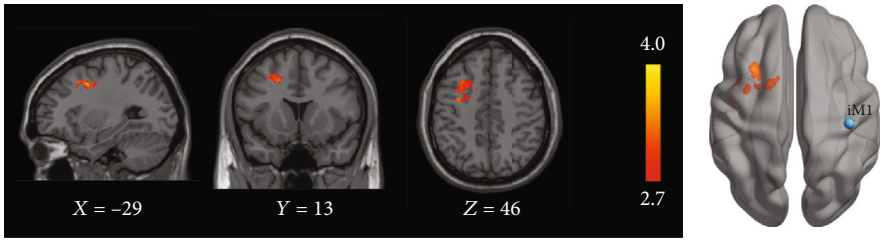
4. Discussion

4.1. Motor Functional Recovery. The reorganization of the central nervous system plays an important role in the recovery of dysfunctions. It is an intrinsic property of the human brain to change its function and reorganize after a lesion forms [35], referred as neuroplasticity in stroke rehabilitation. Leveraging the mechanism of neuroplasticity, robot-assisted hand, which performs high-frequency movements accompanied by sensory feedback, has been shown to be an important factor in improving hand function [36]. The robotic hand could provide haptic as well as proprioceptive feedback on the intended movement. On the other hand, BCI is able to offer feedback to facilitate the appraisal of performance by enforcing the sensory aspect in the sensorimotor loop [37], thereby restoring the action-perception coupling. Some studies have observed significant improvement of FMA scores in BCI groups, but not in the control groups that receive random functional electrical stimulation (FES) [38] or receiving random robotic orthosis feedback [39]. Our study also showed the consistent significant FMA improvement after intervention involved BCI. Besides, the observed increasing trend of success rate also provided the evidence implying that the function of patients improved with more training sessions. In this context, a number of functional brain regions are expected to be involved in the process of recovery. It should be noted that not only perilesional but also distant brain regions would be affected even if the brain damage is focal [40, 41]. Hence, finding the

Table 3: Brain regions showing significant pre-post partial correlation change.

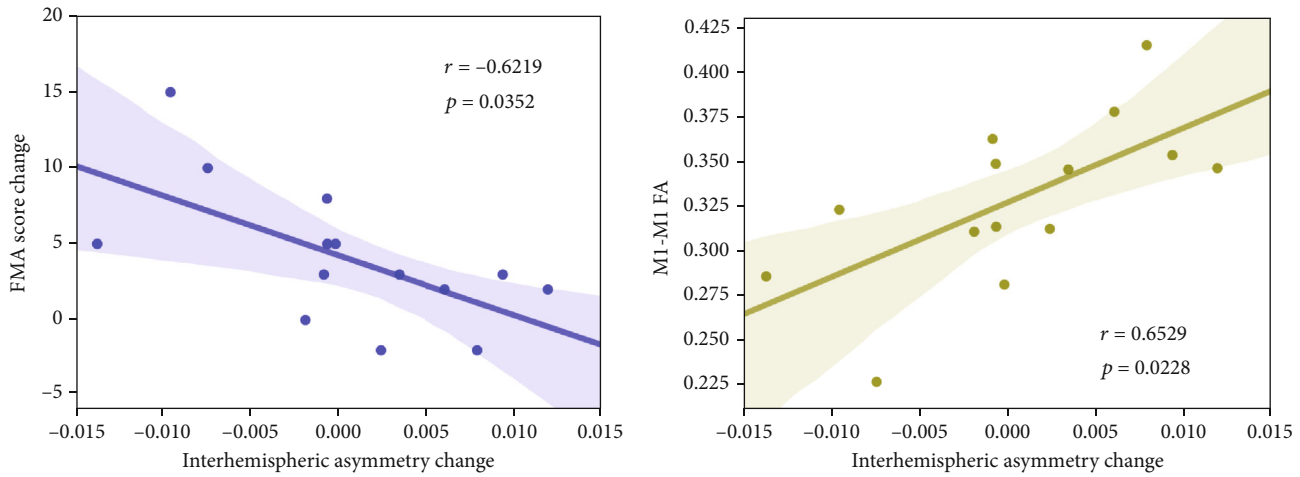
Frequency band	+/-	C/I	Anatomical region	Peak MNI coordinate (x, y, z)		
iM1 regressor						
Theta	+	C	Superior parietal gyrus	-32	-62	40
		C	Inferior parietal gyrus			
		C	Precuneus			
	-	I	Precentral gyrus	52	-16	26
		I	Supramarginal gyrus	46	-11	49
Alpha	+	C	Superior frontal gyrus	-24	18	42
		C	Middle frontal gyrus			
		C	Precuneus	-8	-86	38
		C	Cuneus			
		C	Superior occipital gyrus			
	-	I	Temporal pole	58	10	0
Beta	+	C	Postcentral gyrus	-54	-14	24
		C	Supplementary motor area	-12	8	56
		C	Superior frontal gyrus			
	-	I	Superior temporal gyrus	67	-20	12

+/-: increased or decreased; C/I: contralesional or ipsilesional.



(a) (b)

Figure 4: The conventional seed-based fMRI results illustrated from (a) sagittal view, coronal view, and axial view and (b) rendering surface. The right side is the ipsilesional side.



(a) (b)

Figure 5: Significant correlations were observed (a) between FMA score change and interhemispheric asymmetry change as well as (b) between interhemispheric asymmetry change and FA of M1-M1 anatomical connection.

regions responding to the effect induced by the training therapy is essential for a better understanding of the underlying mechanism of stroke recovery.

4.2. Related Functional Brain Regions. EEG oscillation has been used as an important index for evaluating brain activity, while different bands of EEG signals reflect various brain activities. EEG theta band has been associated with cognitive processing [42] and sensory stimulus identification and codification [43]. Alpha band oscillation is regarded as the dominant oscillatory activity of the human brain and has been associated with basic cognitive functions such as attention and memory [44]. Beta band oscillation is associated with a variety of processes, including top-down communication [45], sensory sampling [46], sensorimotor integration [47], and attention [48]. Simultaneous EEG-fMRI has been widely adopted to investigate how changes in electrophysiological oscillations may be linked to hemodynamic functional interactions within and between brain networks [49, 50].

In our study, increased correlations with BOLD signal were observed in parietal regions for both theta and alpha band EEG signals from iM1 and in prefrontal regions for alpha band EEG signal from iM1. These regions overlapped with the well-known frontoparietal attention network, including portions of the lateral prefrontal cortex and posterior parietal cortex (illustrated in Figure 6(a)). The frontoparietal network is thought to be involved in a wide variety of tasks by initiating and modulating cognitive control abilities [51] and also regulating among default mode network, dorsal attention network, and central-executive networks [52, 53]. Previous simultaneous EEG-fMRI studies have reported that theta and alpha power from EEG correlated with BOLD signals from frontoparietal networks encompassing brain regions involved in an attention process [50, 54]. Meanwhile, it has been reported that brain regions in the frontal-parietal network are highly related to motor imagery BCI training [55] and correlate with the performance of MI-BCI [56]. Increased correlations with the BOLD signal were also observed in sensorimotor areas for beta band EEG signal from iM1 in our study (illustrated in Figure 6(b)). Mantini et al. indicated that the sensorimotor network is primarily associated with beta oscillations [50].

Interestingly, the observed increased correlations were all located in contralesional hemisphere, which suggested the crucial role of interactions between hemispheres, especially motor-related regions during a recovery process. A similar interhemispheric connectivity increase was also seen in fMRI studies. Longitudinal studies indicated that interhemispheric functional connectivity could predict motor improvements after stroke [15, 57]. Pichiorri et al. illustrated the more significantly increased interhemispheric connections between the ipsilesional motor area and contralesional frontal and parietal areas from the beta band of resting EEG data in the BCI-supported MI training group compared with the MI-only group, which were speculated as related to BCI training effects [58]. The similar increased interhemispheric partial correlation found in our study might also be related to BCI training effect. Furthermore, decreased correlations were observed in the ipsilesional supramarginal gyrus and superior temporal gyrus across all the frequency bands and in the ipsilesional precentral gyrus for theta band. It is also interesting to note that all corresponding regions were located in the ipsilesional hemisphere, which might be due to the close distance to the lesion, which implied the functional potential of intrahemispheric communication among ipsilesional regions, consistent with some previous studies [59]. Combining the regions found in the contralesional hemisphere, it seems to provide some evidence that impaired and intact hemispheres might play plausibly complementary roles in responding to training intervention, which deserves further investigation in the future.

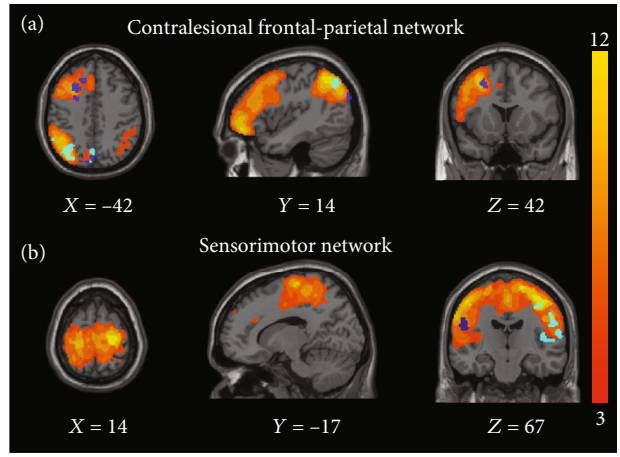


Figure 6: The overlap with contralesional frontal-parietal network and sensorimotor network. (a) The orange color-coded areas indicated the contralesional frontal-parietal network. The azure and violet color-coded areas indicated the regions which showed significant partial correlation change for theta and alpha band EEG signals from iM1. (b) The orange color-coded areas indicated the sensorimotor network. The azure and violet color-coded areas indicated the regions which showed significant partial correlation change for theta and beta band EEG signals from iM1. The contralesional frontal-parietal network and sensorimotor network were extracted using independent component analysis (ICA), and the detailed description of the extraction process is provided in supplementary materials. The right side is the ipsilesional side.

For comparison, the conventional seed-based fMRI connectivity analysis was also conducted. It could be seen that most of the significant functional regions illustrated by the conventional seed-based fMRI connectivity analysis could also be detected by EEG-informed fMRI analysis whose regressors were reconstructed from EEG source signals in our study. Specifically, more functional regions derived from our proposed method were revealed to be influenced by the training intervention. We inferred that the reason for this phenomenon should be linked to high temporal resolution of EEG signal. This allowed more useful neural information to be disentangled from different frequency bands because there are abundant oscillatory activities in the human brain, which could provide a comprehensive understanding of the involvement of functional regions during the recovery process. It is worth noting that as we expected, the significant changes of the partial correlation with SMA were observed. The enhanced partial correlation between contralesional

SMA and ipsilesional M1 was found in both conventional and EEG-informed fMRI analyses. Park and colleagues have shown that functional connectivity of ipsilesional M1 and contralesional SMA at onset was positively correlated with motor recovery at 6 months after stroke, which suggested the significance of preservation of such partial correlation [15]. Therefore, BCI-guided robot hand training might facilitate in restoring and enhancing the communication between contralesional SMA and ipsilesional M1, which might be beneficial for stroke motor recovery later.

4.3. Training Effect Correlated with Interhemispheric Interaction Change. It could be noted that the obvious pattern of interhemispheric interaction from a previous fMRI analysis result existed. We expected that such interhemispheric response should be closely related to training effect. There is a growing awareness about linking the potential motor function improvement after rehabilitation therapies with neural characteristics derived from electrophysiological signals to unfold the underlying mechanisms of the stroke recovery and treatment gains. The majority of these studies were focusing on linear indices, while nonlinear methods have drawn more attention recently. It has been indicated that the human brain is a nonlinear system [60], which cannot be comprehensively explained solely by linear analysis. The complex fluctuations of brain signals are not purely random but reveal a temporal organization over multiple time scales [61]. Hence, nonlinear methods have been proven to be efficient tools in understanding the complexities of the brain, and the measurement of EEG complexity could be linked to the efficiency of brain functional abilities [61]. On the other hand, different from fMRI, EEG has a higher temporal resolution and contains abundant nonlinear dynamic properties [62, 63]. Therefore, a variety of nonlinear methods have been applied to EEG analysis [64].

It was also widely believed that the presence of lesion following stroke would lead to an interhemispheric imbalance where iM1 no longer inhibited cM1 and the normal mutual communication between two hemispheres was severely broken, which has shown to positively correlate with motor impairment [65]. Therefore, the rebalance of the two hemispheres is essential for stroke rehabilitation and stroke recovery. It has been illustrated in some neuroimaging researches that increased change in resting-state functional connectivity of bilateral M1 coupled with better motor and functional improvements after robot-assisted bilateral arm therapy [23]. Pellegrino et al. found interhemispheric correlation changes correlated closely with the acquisition of more accurate hand control after robotic therapy [66]. Consistent with previous studies, our study also demonstrated the significant correlation between FMA score increment and decline in interhemispheric asymmetry, which indicated that more rebalance would bring about more motor improvement.

4.4. Structural Substrate of the Bilateral M1-M1 Connection. Due to the significance of interhemispheric rebalance in the recovery process, we hypothesized that such interhemispheric asymmetry change after training therapy should be built on some structural base in human brains, and the most intuitive one was the interhemispheric structural connectivity via transcallosal commissural projections. Hence, we further explored the association between corresponding asymmetry change and M1-M1 anatomical connection and found that the interhemispheric asymmetry change significantly correlated with the FA value of M1-M1 connection fibers, which indicated that more interhemispheric rebalance could be achieved for patients with lower M1-M1 anatomical connection. In a recent study, it was observed that, among stroke patients with good motor outcomes, those with more severe impairment in M1-M1 anatomical connection had a higher M1-M1 resting-state functional connectivity [67] which implied the importance of restoring interhemispheric interaction for patients with lower M1-M1 connection level to achieve ultimate recovery goal. Together, these findings suggested that our training intervention protocols or similar therapies should be considered, especially for patients with poor M1-M1 anatomical connection.

4.5. Limitations and Future Work. Several limitations need to be noted in the current study. First of all, the sample size was not large, which might limit the generalization power to some extent. Second, most of our subjects were male which might restrict our finding extended to female stroke population although we assumed that the gender factor was less likely to affect the result significantly. Another potential concern was about the influence of the stroke lesion on the reconstructed source data. It should be noted that, in the current cohort of stroke subjects, most lesions were located in the subcortical regions. Because of this, we limited the source space to the cortex when performing the EEG distributed source estimation which was also widely adopted in practice [68]. Meanwhile, we mainly extracted source signals from iM1 and cM1 seeds which were also far away from the lesion regions. Besides, the 64-channel EEG set-up could already achieve an accurate description of the spatial distribution of the stroke-related EEG, which guaranteed the quality of the source estimation to some extent [69]. Hence, in our study, the influence of the stroke lesion on the quality of the reconstructed source data was supposed to be negligible. However, more advanced algorithms that take the brain lesions into account could be developed for more accurate source estimation in future studies. Besides, due to the lack of the control condition, it is quite difficult to check whether and to what extent the observations were clearly linked to our experimental intervention. Therefore, a control group with pure robot hand training without BCI could be included to clarify this vagueness in the future.

Recently, studies have proposed that surface-based methods might improve the quality of cortical area localization compared to the volume-based methods in fMRI analysis [70]. However, it should be noted that studies on EEG-informed fMRI with a surface-based method are quite scarce, compared with a volume-based method. We also tried a preliminary attempt on EEG-informed fMRI analysis with surface-based method. The detailed analysis process and the observed results were described in supplementary materials. Furthermore, a more standard pipeline of EEG-informed fMRI analysis with surface-based method should be developed to fill this gap in the future.

5. Conclusion

This study presented a paradigm to investigate the neural correlates of motor recovery after training therapy based on multimodality neuroimaging techniques, which could provide more complementary information for each other such as oscillatory information derived from EEG signal. Some significant brain regions linked to important functional networks were observed to be sensitive to our integrated BCI-guided robot-hand training intervention, although cautions should be taken when interpreting these observations due to the absence of a control group. The training effect was found to be highly related to interhemispheric asymmetry alternation. The underlying structural substrate might be associated with M1-M1 anatomical connection. Finally, our study provided valuable clinical information for both stroke prognosis and understanding of regional communication in the brain given training therapy.

Data Availability

The data generated for this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Cheng Chen and Kai Yuan contributed equally to this work.

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Supplementary Materials

Supplementary materials consist of section 1—Scheme of Experimental Protocol and Photograph of the Robot Hand, section 2—Data Analysis, section 3—Extraction of Contralesional Frontal-Parietal Network and Sensorimotor Network, section 4—Interhemispheric Asymmetry before and after Training, and section 5—EEG-Informed fMRI Analysis using Surface-Based Method. *(Supplementary Materials)*

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Research Article

Sensorimotor, Attentional, and Neuroanatomical Predictors of Upper Limb Motor Deficits and Rehabilitation Outcome after Stroke

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The rehabilitation of motor deficits following stroke relies on both sensorimotor and cognitive abilities, thereby involving large-scale brain networks. However, few studies have investigated the integration between motor and cognitive domains, as well as its neuroanatomical basis. In this retrospective study, upper limb motor responsiveness to technology-based rehabilitation was examined in a sample of 29 stroke patients (18 with right and 11 with left brain damage). Pretreatment sensorimotor and attentional abilities were found to influence motor recovery. Training responsiveness increased as a function of the severity of motor deficits, whereas spared attentional abilities, especially visuospatial attention, supported motor improvements. Neuroanatomical analysis of structural lesions and white matter disconnections showed that the poststroke motor performance was associated with putamen, insula, corticospinal tract, and frontoparietal connectivity. Motor rehabilitation outcome was mainly associated with the superior longitudinal fasciculus and partial involvement of the corpus callosum. The latter findings support the hypothesis that motor recovery engages large-scale brain networks that involve cognitive abilities and provides insight into stroke rehabilitation strategies.

1. Introduction

Stroke survivors may suffer from motor, cognitive, and/or psychological deficits, with conjoined consequences for the course of rehabilitation as well as for the quality of life. The presence of motor impairments (i.e., hemiparesis, coordination problems, and spasticity) is very common and it evidently affects patients' everyday autonomy, with a high variability of recovery that depends on both spontaneous and rehabilitation-induced gains [1].

The rehabilitation of neurological motor impairments is based on motor learning principles within complex sensorimotor and cognitive processes [2]. Repracticing the execution of goal-directed actions requires some planning and computational steps that engage connections among various brain areas [3, 4]. This hierarchical process goes from the sensory integration between bodily information learned from previous experiences [5] and on-line movements and context [4, 6] up to the execution of voluntary movements. On one side, the interpatients variability in preserved sensorimotor abilities is critical for functional motor skills [7], on-going control [8], and prognosis [9]. On the other side, the cognitive system supports motor execution, in terms of planning the computational steps and of attention on internal and external sensorimotor feedbacks to monitor and adjust the performance [6, 10, 11]. As a matter of fact, stroke patients with motor deficits mainly have difficulties to cope with everyday actions, which often involve high attentional load due to multitasking demands (e.g., walk and avoid obstacles), thereby worsening sensory inputs' processing [12] and motor

execution [13]. Indeed, the major goal of motor rehabilitation is the recovery of everyday life abilities.

Recent innovative approaches for motor rehabilitation with technology-based (hereafter, TB) techniques aim to resemble the ecological environments, where behavior is demanding and cognitive abilities may be involved [2, 14]. TB methods are based on interactive action-feedback simulation software, which engages patients into real-world-like scenarios [2, 15, 16] and supports motor recovery, as demonstrated for upper limb rehabilitation [17–20]. Nevertheless, a recent Cochrane review noted that most studies of TB rehabilitation (i.e., using virtual reality) usually exclude patients with severe cognitive deficits, thereby prompting for further investigations on cognitive abilities as covariate in motor training outcome [21].

Considering the integration of motor and cognitive systems underlying motor learning [2], a crucial challenge is to exploit their functioning at a neural level in neurological patients. It is well known that lesions in primary and secondary motor cortices [2], corticospinal tract [22], and interhemispheric connections [23] affect the severity of upper limb impairments. However, recent results highlight the role of brain connectivity encompassing bilateral motor, premotor, and frontal areas [24] and forming a large-scale temporofrontoparietal functional network [25–28]. The neural plasticity of this large-scale network may give insight into the interpatients variability in motor recovery [29, 30] within the cooccurrence of cognitive deficits [31]. In particular, a clear link between motor and attentional abilities is shown by the neglect syndrome [32, 33], a visuospatial attention deficit in orienting and reporting relevant stimuli on the contralesional side of space [34], mainly occurring after right hemisphere stroke ([35, 36], but see [37]). More generally, the efficacy of motor rehabilitation may depend on many factors that include patients' residual abilities [1, 9], training approaches [15], and type of neuroanatomical impairments [3, 38].

The goal of the present retrospective study was to investigate how the sensorimotor and attention systems contribute to motor recovery of upper limb impairments following TB rehabilitation. We only considered patients who underwent a TB physiotherapy program in order to have a consistent rehabilitation approach, which was also closer to real-life requests. We examined the influence of selective attention skills in the whole sample of patients, whereas for a subgroup of right stroke patients we additionally examined the role of visuospatial orienting abilities. To complete the picture, we also inspected the neural structures associated with both initial and postrehabilitation motor performance. We examined the association with the structural lesion [39] as well as with the white-matter disconnections [40]. The latter represents a novel approach to examine direct structural disconnections after a focal lesion [40] and provides valuable knowledge about the mapping between connectivity and behavior [24].

2. Materials and Methods

2.1. Participants. Stroke patients hospitalized between 2010 and 2017 at IRCCS San Camillo Hospital (Venice, Italy) were considered for the retrospective study.

Patients were initially inspected for the following features: adult age, first stroke (from ischemic or hemorrhagic etiology), and availability of a brain structural MRI scan. Consequently, inclusion criteria were applied for (1) presence of unilateral brain lesion, (2) completion of upper limb TB rehabilitation protocol, and (3) administration of the attentive matrices test [41]. Additional exclusion criteria were implemented to take in consideration only patients who were likely to benefit from the motor rehabilitation: (1) presence of other neurological and psychiatric conditions in medical history assessed by available neurological tests and/or brain MRI scan (i.e., clinical signs of probable neurodegenerative deficits), (2) chronic stroke lesion (>1.5 years from onset), (3) pretreatment motor function of the upper limb showing negligible (values at the Upper Extremity Fugl-Meyer Assessment scale in the range 60-66, for potential ceiling effect) or very severe impairments (values in the range 0-6, for potential floor effect), which could impact the scale's sensitivity [42], and (4) long distance (>3 months) between assessment of attention and TB rehabilitation treatment.

From the primary eligibility screening, 42 patients satisfied all the inclusion criteria, but other 13 patients were ruled out for exclusion conditions. The final sample consisted of 29 patients (mean age = 62.41 ± 11.87 years, mean education = 11.41 ± 4.50 years, mean time from onset = 7.18 ± 4.60 months), 11 with left (LBD) and 18 with right brain damage (RBD) (see Figure 1 for study inclusion flowchart; complete patients' data are provided in Supplementary materials). The study adhered to the Declaration of Helsinki and to the Italian regulation (Legislative Decree n. 211/2003; Ministry Decree 17 December 2004) for experimental studies in health care. The Ethical Committee for Clinical Research of the IRCCS San Camillo Hospital approved two studies to enroll patients after informed consent (Prot. 2013.11, registration at ClinicalTrials.gov NCT02234531 with virtual reality, and Prot. 2014.14 – sERF, registration at ClinicalTrials.gov NCT03207490 with AMADEO).

2.2. Cognitive Data. All patients underwent a neuropsychological assessment, but not consistently for the whole sample due to the retrospective design of the study. For a description of the sample, we recorded the tests present for at least 50% of the patients. These tests explored the following: general cognitive abilities (Minimental scale examination—MMSE [43]), reasoning (Raven's progressive matrices; [44]), short-term memory (Forward digit span, Spinnler and Tognoni, 1987), long-term memory (Rey figure - delayed; Caffarra et al., 2002), working memory (Backward digit span, [41]), and constructive apraxia with simple and complex figures (Copy of drawing; Spinnler and Tognoni, 1987; Rey figure - copy; [45]).

For the purpose of exploring attentional influences on motor rehabilitation responsiveness, we collected attentional test data. Selective attention was evaluated by the attentional matrices test, which is suitable for examining both RBD and LBD stroke patients [41]. In this test, patients are required to cross out some target numbers (1, 2, or 3) in three different numerical matrices within 45 seconds (overall range

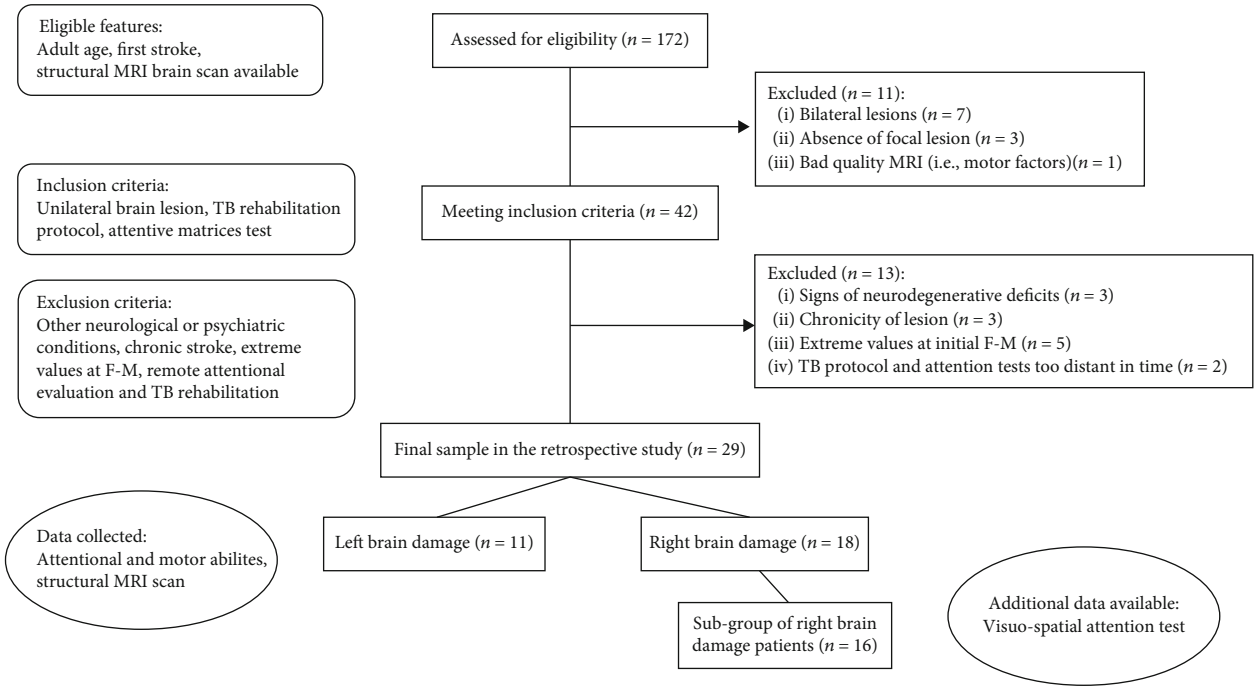

Figure 1: Enrollment flowchart. MRI: Magnetic Resonance Imaging; TB: Technology-based; F-M UE: Fugl-Meyer Upper Extremity test.

0-60). Additionally, the assessment of visuospatial attention through the Behavioral Inattention Test (BIT) [46] was available for almost all of the right brain-damaged patients (16 out of 18 patients). The BIT includes 6 subtests (cancellation of lines, letters or stars, line bisection, figure copy, and drawing) to evaluate difficulties in visuospatial attention, and it is routinely used to assess the presence of neglect. BIT subtests highlight slightly different types of neglect, but only the cancellation tasks directly require visual scanning in the peripersonal space [47]. In particular, the Stars cancellation subtest requires to mark the little stars (range 0-54) in a page of confounders (big stars and words), thereby complicating visual scanning performance to yield a sensitive evaluation of neglect. Performance in the BIT Stars test was therefore used for the statistical analyses.

2.3. Motor Data. All the patients completed a physiotherapy rehabilitative program, which consisted of two different trainings: a traditional rehabilitation (TR) treatment and an additional one with TB technologies. Each treatment lasted for 1 hour/day for 5 sessions (3 weeks), 30 h overall. Both trainings were tailored to the patient's motor residual capabilities with progressive exercises' targets. Their combined responsiveness (TR+TB) was tested by the Upper Extremity section of the Fugl-Meyer Assessment scale.

Specifically, the TR exercises targeted the whole body to improve the patient's autonomy. The specific exercises for the upper limb consisted of passive, assisted, and active mobilizations in all free directions [48], driven by the physiotherapist. The TB rehabilitation protocol focused only on the upper limb's exercises in an ecological virtual setting, with the support of technologies that provided on-line reinforcement feedbacks. The protocol could use virtual reality software or the AMADEO robot, which are specifically applied for the rehabilitation of the upper limb with slight differences for the trained muscular districts. The exercises with virtual reality focus on the elbow and shoulder/proximal upper limb, with a 3D motion-tracking system (Polhemus 3Space FasTrak, Vermont, U.S.A.) as described by Piron and colleagues [2]. The AMADEO robot (Tyromotion GmbH Graz, Austria) treatment is based on detection and control of fingers' flexors and extensors through surface electromyography signals [19]. The choice of TB protocol was driven by clinical judgment and in particular by the residual abilities of the individual patient.

For the evaluation of sensorimotor abilities, all patients underwent a complete clinical assessment before treatment by (i) Modified Ashworth scale [49], for spasticity of five upper limb's muscle (total value was computed as the sum of each muscle, ranging from 0 to 20 as increasing severity), and (ii) Reaching Performance scale (range 0-36) ([50], for the upper limb reaching abilities. Additionally, the use of the Fugl-Meyer (F-M) scale [42] was considered separately for (iii) Sensation (range 0-24), rating impairment of tactile and proprioception sensation; (iv) Joints amplitude (range 0-48) rating range of motion and pain associated with passive mobilization of the upper limb; and (v) Upper Extremity (UE) (range 0-66) for overall assessment of upper limb motor function. The F-M UE subscale was readministered after rehabilitation as the primary measure to register possible changes between pre- and post-treatment performance [51].

2.4. Brain Lesion and Disconnection Preprocessing. All patients had a T1-weighted image from a 1.5 T Philips MRI scanner. As a first step, automated brain lesions segmentation was obtained using the Lesion Identification with Neighborhood Data Analysis software (LINDA [52]). The resulting lesion mask (in native MRI space) was visually inspected and

manually corrected with ITK-snap software [53] by two researchers (RZ and DE) and the supervision of a neurologist in the case of slight differences between LINDA results and original T1 scans. Finally, to allow comparisons across patients, the lesion was spatially registered to a standard template using the pipeline of the BCBtoolkit software (http://toolkit.bcblab.com/) [40] (also see [24]). The individual lesion was replaced with healthy tissue of the contralateral hemisphere in an enantiomorphic method [54] to allow MRI scans and lesion masks' normalization to a MNI152 space (with $2 * 2 * 2$ millimeters voxel size) with diffeomorphic deformation [55]. A quality check on the registration step was carried out through visual inspection.

The probable lesioned tracts were extracted using the BCBtoolkit Disconnectome maps tool [40]. In this approach, the individual lesion map was used as a seed for the tractography in TrackVis (http://trackvis.org/), by taking into account the interindividual variability from a healthy controls' dataset (as in [56]). In the resulting disconnections maps, voxels represent only disconnected tracts above the conventional probability threshold of 50% [40]. Note that values in the maps correspond to the maximum lesioned-streamline localization probabilities, not disconnection probabilities.

3. Data Analysis

3.1. Behavioral Data Analysis. In order to control descriptive difference across the sample, a first direct comparison between patients was run in relation to the side of lesion (LBD vs. RBD) on available neuropsychological assessment and experimental data (i.e., demographic, neurological, motor, and attentional), by means of T-test or Wilcoxon Test for continuous and ordinal data or Chi^2 test for frequencies.

The inspection of motor rehabilitation responsiveness was run on the F-M UE outcome. Previous studies have shown that the initial severity of deficit is predictive of the behavioral recovery [57, 58]. Therefore, we computed a "F-M UE recovery index" [((posttreatment F – M UE – pretreatment F – M UE)/pretreatment F – M UE) $*$ 100] to detect motor changes weighted by the pretreatment residual performance [57, 58]. Note that a raw measure of change (i.e., post–pre) does not consider the patient's initial ability and it would miss its impact on the performance gains. After controlling that its distribution did not diverge from normality using the Shapiro-Wilk test, this index was used as a dependent variable to analyze the association of motor improvement to all other collected data by means of a linear regression model. As in previous studies with a similar goal [59], a forward stepwise approach permits to sequentially introduce the variables in accordance with correlations to the dependent variable (the full correlation matrix is reported in Tables 5S-6S in Supplementary materials). The model fit was assessed by log-likelihood tests to compare models' residuals by Chi^2 tests (entering those with $p < 0.10$), including all those factors that help explaining variance, but do not prevent model convergence [60]. Moreover, the robustness of the stepwise regression results was assessed using an alternative method, the best subset regression [61]. The latter generates models from all possible predictors' combinations, which are then compared in terms of goodness-of-fit. These results are reported in supplementary materials. Notably, the most conservative model contained the same predictors as the stepwise regression.

In the model, associations to the F-M UE recovery index were computed for the following independent variables: demographic information (i.e., age, gender, and education), clinical parameters (i.e., etiology, time from onset, damaged hemisphere, lesion volume, and type of TB motor training), attentional deficits (values at the attentional matrices test), and pretreatment upper limb residual motor performance. For the latter, a dimensionality reduction was carried out using principal component analysis (PCA) with oblique rotation on all collected pretreatment motor tests (i.e., Modified Ashworth, Reaching test, and all F-M subscales). Following Corbetta and colleagues (62; also see [24, 57]), we used the first principal component as "motor factor" score in all subsequent analyses, as it accounted for most of the variance (>60%, see Supplementary materials). The motor factor score is therefore highly representative of the motor tests and its use for regression modeling prevents the problem of including several correlated tests as predictors.

We also carried out an exploratory analysis to investigate the role of visuospatial attention in a subgroup of RBD patients for whom the BIT Stars score was available (16 out of 18). This test evaluates the visuospatial orienting component of attention, which is more frequently impaired following right hemisphere stroke ([36], but see [37]) and might be a better predictor of motor recovery compared to the more general index of selective attention available for the whole sample. The BIT Stars score was entered as an additional predictor variable in the regression analysis.

Analyses were run using the software R (R Core Team, 2018), using the package car [62].

3.2. Neuroimaging Data Analysis. To overcome the problem of small sample size, the lesion data were aligned onto a single hemisphere by flipping left lesion masks and disconnection maps into the space of the right hemisphere.

Firstly, an overlay map was created separately for lesions and disconnections. These maps allow us to depict the most overlapped damaged areas and to describe their localization. Afterwards, statistical analyses were separately run for lesions masks and disconnection maps, with a voxel-based lesion mapping (VLSM) method [39, 63] using the NPM program in the MRIcron software (http://www.cabiatl.com/mricro/mricron/index.html). The VLSM approach permits to explore strong lesion-deficit associations within a small neurological sample [64], by independently comparing all damaged voxels in a mass-univariate design [65, 66].

Two separate VLSM analyses were computed to estimate damaged voxels that predict the lower values at pretreatment F-M UE and at F-M UE recovery index, both for grey matter lesions and white matter disconnections. For instance, VLSM results report the damaged areas associated with residual abilities and motor recovery, respectively. Analyses were run using nonparametric Brunner-Menzel (BM) analysis on

Table 1: Values for whole sample and divided for damaged hemisphere.

Test	Total	LBD	RBD	LBD vs. RBD comparison
Sensorimotor abilities				
Pretreatment F-M UE	32.07 ± 16.16	41.73 ± 16.24	26.17 ± 13.32	$p = 0.015$ *
Posttreatment F-M UE	38.52 ± 17.52	48.82 ± 16.39	32.22 ± 15.39	$p = 0.013$ *
F-M UE recovery index	24.01 ± 21.44	20.60 ± 12.68	26.10 ± 25.50	$p = 0.446$
Modified Ashworth	3.55 ± 3.62	1.09 ± 2.21	5.05 ± 3.52	$p = 0.002$ *
Reaching performance	17 ± 12.73	24.45 ± 11.85	12.44 ± 11.24	$p = 0.011$ *
Sensation	18.48 ± 6.43	20.82 ± 4.31	17.05 ± 7.18	$p = 0.220$
Joint amplitude	40.83 ± 6.08	42.64 ± 5.70	39.72 ± 6.20	$p = 0.182$
Type of TB (% virtual reality)	79.31%	81.82%	77.78%	$p = 0.238$
Attentional abilities				
Attentional matrices	38.31 ± 12.86	39.09 ± 11.48	37.83 ± 13.94	$p = 0.794$
BIT Stars			45.64 ± 14.11	

Note: Patients with LBD: left brain damage; RBD: right brain damage; F-M UE: Fugl-Meyer Upper-Extremity Fugl-Meyer test; p: p value; *: significant result.

Table 2: Significant regression model.

Independent variables	Est. Coeff.	St. Coeff.	Std. Err.	t value	p value
Intercept	-0.382	-0.382	-0.199	-1.992	0.0.058′
Age	0.013	0.722	$2.609e^{-3}$	4.997	<0.0001***
Lesion volume	$-3.915e^{-6}$	-0.240	$-2.048e^{-6}$	-1.912	0.068′
Affected hemisphere	-0.198	-0.457	-0.069	-2.858	0.009**
Motor factor	0.142	0.662	0.031	4.491	0.0002***
Attention	$4.487e^{-3}$	0.269	$2.143e^{-3}$	2.094	0.047*

Note: Est. Coeff.: estimated coefficient; St. Coeff.: standardized coefficient; Std. Err.: standard error. Affected hemisphere is coded as 1 = left and 2 = right. p values: *** <0.001, ** <0.01, * <0.05, ′ <0.10.

each voxel within the lesion mask for continuous behavioral data [67], controlling for lesion volumes as covariate, with voxel-level false discovery rate correction for multiple comparisons. With an atlas-based approach for identification [68], VLSM results were overlapped to the probabilistic Harvard-Oxford atlas [69] and the human brain atlas for single tracts [70] to label and identify the damaged voxels in grey structures and white matter tracts (see Supplementary materials for details).

4. Results

4.1. Descriptive Information. In order to ensure comparable groups, all relevant behavioral data were compared between LBD and RBD patients. RBD patients reported lower motor abilities in pre- and post-treatment assessments, but not in the F-M UE recovery index (Table 1). Other neuropsychological data was not available for the whole sample but is reported in the supplementary materials for descriptive purpose (Table 3S).

4.2. Motor Rehabilitation Responsiveness. The F-M UE recovery index did not diverge from a normal distribution (Shapiro-Wilk test, $W = 0.949$, $p = 0.102$), which is appropriate for linear regression modeling. In the resulting model, the F-M UE recovery index was predicted by age ($p < 0.001$), motor factor ($p < 0.001$), affected hemisphere ($p = 0.009$), and attentive matrices ($p = 0.047$) (see Table 2). No significant relation to other independent variables was found. The model yielded a very good fit, with $R^2 = 0.661$ (adjusted $R^2 = 0.587$, F – statistic's Test = 8.969, $p < 0.001$). The residuals of the model did not diverge from a normal distribution ($W = 0.957$, $p = 0.273$).

An additional regression analysis on pretreatment F-M UE scores, reported in supplementary materials, revealed that initial motor performance was only influenced by time from stroke onset.

The F-M UE recovery index for the subgroup of right hemisphere stroke patients was still not statistically different from a normal distribution ($W = 0.894$, $p = 0.065$). Regression modeling showed that the recovery index was predicted by age ($p < 0.001$), time from onset ($p = 0.034$), and BIT Stars test ($p = 0.033$) (Table 3). No other predictor was significant. The model yielded $R^2 = 0.713$ (adjusted $R^2 = 0.641$, F – statistic's Test = 9.936, $p = 0.001$), and the residuals did not diverge from a normal distribution ($W = 0.944$, $p = 0.399$).

Table 3: Significant regression model for the subgroup of right brain damage patients.

Independent variables	Est. Coeff.	St. Coeff.	Std. Err.	t value	p value
Intercept	-1.417	-1.417	0.318	-4.462	0.0008***
Age	0.018	0.891	0.003	4.973	0.0003***
Time from onset	0.022	0.429	0.009	2.389	0.034*
BIT Stars	0.007	0.374	0.003	2.412	0.033*

Note: Est. Coeff.: estimated coefficient; St. Coeff.: standardized coefficient; Std. Err.: standard error; p values: *** <0.001, * <0.05.

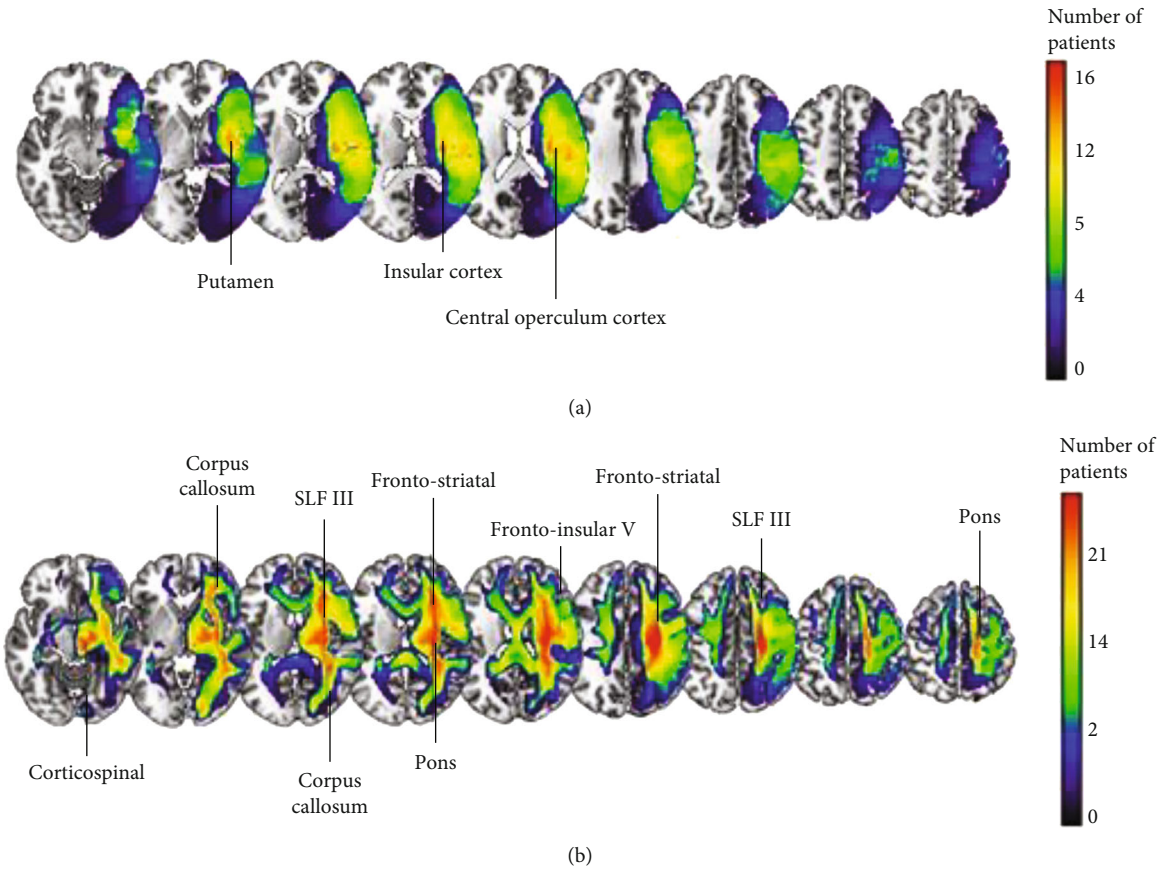

Figure 2: Overlay maps of lesions (a) and white-matter disconnections (b) on a standard brain MNI template. The color scale represents the number of patients.

4.3. Lesion and Disconnection Data. Maximum lesion overlap was found in 17 patients (58.62%), and it mainly involved putamen, insular, temporal, and central operculum cortices (Figure 2(a)).

In terms of disconnected tracts, maximum overlap was found in 25 patients (86.21%). The most damaged tracts in percentage across all patients were corticospinal tract, corpus callosum, corticopontine, frontostriatal, frontoinsular tract V, and superior longitudinal fasciculus III (SLF III) (Figure 2(b)).

4.4. Predicting Motor Abilities and Recovery from Neuroanatomical Data. In VLSM analysis, lower pretreatment motor performance was significantly associated with clusters of damaged voxels mainly located in putamen and insular cortex (Figure 3(a)), as well as to white matter disconnections within corticospinal tract, corticopontine, frontostriatal, and frontoinsular tract V (Figure 3(b), see Table 9S for detailed results in Supplementary materials).

In the VLSM analysis for motor rehabilitation responsiveness, lower F-M UE recovery index was significantly associated with a wide parietal region. Even though significant results emerged in the lesion analysis for a small cluster located around the central gyrus, they were present in less than 50% of patients. In contrast, the white matter was found to be more reliably involved in motor outcome, especially across the SLFIII and the corpus callosum (Figure 4, see Table 10S for detailed results in Supplementary materials).

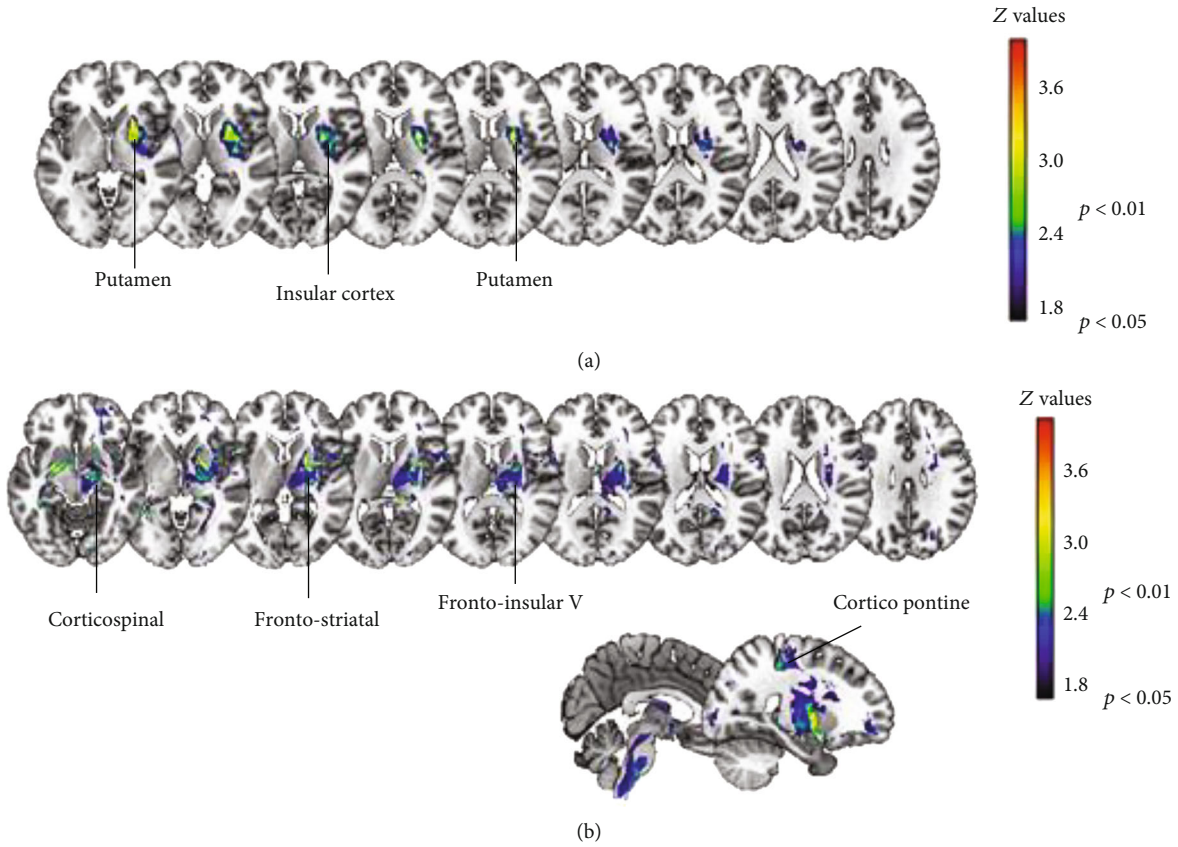

Figure 3: Significant brain-behavior associations observed between the pretreatment F-M UE scores and lesions (a) or white-matter disconnections (b).

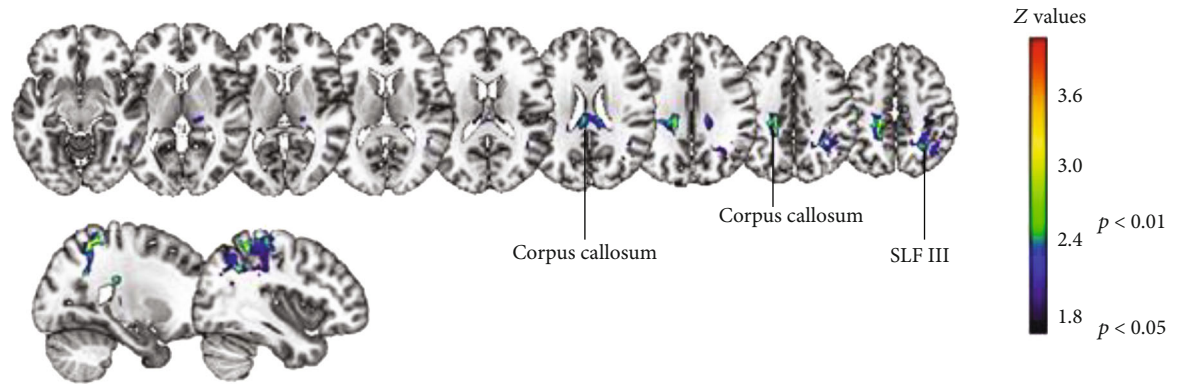

Figure 4: Significant brain-behavior associations observed between F-M UE recovery index and white-matter disconnections.

5. Discussion

In the neurological population, the rehabilitation of motor deficits relies on both sensorimotor and cognitive systems [2]. Voluntary motor behavior involves a wide neural network beyond motor [24, 28, 57] and attentional functions [32, 33]. However, the integrated investigation of motor, cognitive, and neuroanatomical factors that may influence motor recovery is still sparse.

The present retrospective study investigated whether attentional abilities influenced the outcome of motor rehabilitation, when controlling for clinical variables and for pretreatment sensorimotor skills. A second aim of the study was to assess which brain lesions and/or white-matter disconnections better predict the motor deficits and hinder the rehabilitation outcome. Even though sample size was small, all patients participated in TB rehabilitation programs for the upper limb in the context of clinical trials. This ensured

consistency in the rehabilitation protocols and the availability of a detailed assessment of motor skills.

5.1. Sensorimotor System and Neuronal Correlates. In the linear regression model, for the whole sample of patients, the F-M UE recovery index was predicted by pretreatment sensorimotor abilities, attention, affected hemisphere, and age. This analysis highlights that some patients' characteristics contribute to interpatients' variability in responsiveness to motor rehabilitation. Notably, the model accounted for a large amount of the variability in the motor recovery index.

It is worth noting that upper limb sensorimotor residual abilities were summarized by the first principal component of a PCA conducted on all motor tests and scales. In line with previous studies that used the same approach [24, 71, 72], we observed that the first component accounts for a large amount of behavioral variance (here 76%). This is consistent with the idea that behavior is low-dimensional [71] and that a single "motor factor" adequately captures the residual motor abilities. Importantly, the motor factor influenced motor rehabilitation outcome. It is also worth noting that motor factor values are more influenced by pretreatment F-M UE and reaching performance scales than by simpler variables like sensation, proprioception, spasticity, and joints amplitude (see Table 4S in Supplementary materials for details). Considering that higher values of the motor factor index poorer motor performance and that the corresponding model regression weight was positive, it can be concluded that the performance gain (relative to pretreatment performance) yielded through rehabilitation was larger for patients with more severe upper limb motor difficulties. This suggests that patients with severe motor deficits have more "room for improvement" and it is consistent with the evidence that TB rehabilitations may boost upper limb motor amelioration [20] even for the most compromised patients.

The VLSM analyses related the patients' pretreatment F-M UE scores to lesions in sensory and motor areas, most notably the putamen and the insula. The putamen is considered as a primary motor structure, which is also necessary for higher-level motor processing, such as in mental rotation that relies on sensory memory and supports new learning [73]. The insula is a crucial area for cognitive processing of bodily awareness [74, 75] through the processing of various sensory internal stimuli [76, 77], but it is also involved in high-demanding attentional tasks and control, thanks to its interaction with large-scale brain networks [78].

In VLSM analyses on disconnection maps, the damage of the corticospinal tract and of some frontoparietal pathways (i.e., corticopontine, frontostriatal, and frontoinsular V tracts) emerged as predictors of the pretreatment motor abilities. The corticospinal tract is part of the main motor pathway, with a major role in controlling voluntary actions [79]. The involvement of other frontoparietal networks may instead suggest associations to other cognitive domains such as attention and language to monitor own motor execution and interact with external stimuli [28].

In relation to the lesion side, descriptive statistics revealed differences in the motor abilities between LBD and RBD patients, with the latter presenting higher severity of upper limb spasticity and reaching performance deficits. Lesion side influenced motor recovery outcome in the model. This might be related to small differences between LBD and RBD in the distribution of lesions affecting the primary sensorimotor systems.

Interestingly, the type of TB therapy did not enter into the model. This is in line with the fact that both TB methods are built on exercises of kinematic adaptation to continuous on-line feedback in ecological settings, as well as with the previous evidence that both methodologies boost upper limb motor recovery [17–20].

5.2. Cognitive System and Neuronal Correlates. Our regression modeling results show that selective attention skills (evaluated by the attentive matrices test) are positively related to the F-M UE recovery index. This result supports the hypothesis that preserved attention skills can positively impact the motor rehabilitation outcome, as motor and attention processes work together in motion [80].

Nevertheless, the complementary regression analysis carried out on the subgroup of patients with right hemisphere lesions suggests that the attentional modulation of the rehabilitation outcome is more specifically linked to visuospatial orienting as opposed to the more general selective attention. Further studies should exploit computerized assessment methods that can unveil more subtle visuospatial orienting deficits [81], even in LBD patients [37]. Spatial abilities are important for motor recovery of both RBD [33] and LBD patients [82], but unfortunately, our data did not include tests exploring other spatial processes such as apraxia [83].

The involvement of visuospatial attention is consistent with the results of the VLSM analysis on the motor recovery index (Figure 4). Indeed, SLF III is relevant in the intrahemispheric frontoparietal network supporting attentional orienting that has been associated with visuospatial neglect [71, 82]. Moreover, SLF III is thought to have a role in the link between the attention to salient stimuli and the planning of goal-directed actions [84]. The involvement of the corpus callosum seems instead to support the role of interhemispheric connectivity in stroke recovery, as previously reported for both motor deficits [57, 81] and visuospatial neglect [85, 86]. From the lesion analysis, a central parietal area was detected, but only in a small number of patients. This area may be linked to neglect severity [87].

Nevertheless, deficits in both motor and attention domains may stem from lesions inducing wide functional changes [72] in frontoparietal and interhemispheric connectivity [86]. Damage in SLF III and corpus callosum may support the idea of a widespread disruption of cortical activity in both motor and cognitive domains, disclosing the cooccurrence of attentional and motor impairments in stroke patients [33]. Indeed, the upper limb motor recovery of voluntary movements in our sample was supported by attention skills, which are also important for the higher-level cognitive processes of monitoring [6] and controlling [3] the motor execution.

In the same vein, SLF III was recently shown to be disconnected in stroke patients with anosognosia for hemiplegia

[74], who overestimate their upper limb motor performance due to a lack of awareness for the motor impairment. Right hemisphere damage to the frontotemporal-parietal network disrupts the computational steps between motor planning and higher-level monitoring [88], affecting the level of awareness [89] and its fluctuations [90]. Even though anosognosia and neglect are mainly investigated in RBD patients, they can also occur following left hemisphere damage [37, 91] and are well known to negatively impact motor and cognitive recovery [92, 93].

Finally, patients' age was a significant predictor in the models, but its effect appears counterintuitive because it associated older age with higher values of the F-M UE recovery index. It should be noted, however, that the mean age was relatively high (62.414 ± 11.879 years) and the results might have been influenced by other demographic or clinical variables (note also that there was no correlation between age and pretreatment motor deficit; see full correlation matrix in Table 5S of the supplementary materials).

5.3. Study Limitation. The main limitation of the study is the relatively small sample size. This is due to the retrospective design and to the fact that patients underwent tailored assessments. This prevented the inspection of a broader range of cognitive domains. Moreover, we only considered variables without missing data in order to examine effects for the whole group and overcome model convergence issues. Similarly, for neuroanatomical analysis, we applied univariate statistical methods as suggested for lesion investigations in small samples, thereby ensuring a high specification in resulting clusters [66]. Despite univariate and multivariate brain-behavior mapping approaches have been shown to produce highly similar results [24], a bigger sample and the use of multivariate machine learning methods would have strengthened the generalization of our findings. Additionally, the severity of disconnections could be estimated more directly using other methods (e.g., [94]). Future studies should exploit a prospective design to collect information on a broader range of sensorimotor and cognitive skills, as well as multimodal neuroimaging data, to predict motor recovery in a large sample of patients.

6. Conclusion

The present retrospective study aimed to integrate clinical, behavioral, and neuroimaging data as predictors of upper limb motor recovery, exploiting a relatively small but selected sample of patients that consistently received TB motor rehabilitation. Results showed that age, hemisphere, pretreatment motor, and attentional abilities are associated with motor rehabilitation outcome. The integration of motor and cognitive variables is crucial to understand patients' variability in rehabilitation. For example, attention deficits, in particular visuospatial orienting, could play a key role into motor recovery of the upper limb, supporting rehabilitation's engagement and final outcome.

Brain-behavior mapping showed that frontoparietal areas are involved in both patients' residual motor abilities and recovery, but with different weighted contributions. While the pretreatment motor performance was more connected to motor areas and pathways, motor rehabilitation outcome was predicted from both motor and attentional networks.

In conclusion, the integration of behavioral and neuroanatomical information is a valuable approach to understand and tailor upper limb motor treatment in stroke patients. The possibility of predicting rehabilitation outcomes might inform clinical decisions on the intervention program, thereby optimizing resources and fostering patients' recovery.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors report no competing interests.

Authors' Contributions

Daniela D'Imperio was responsible for the methodology, formal analysis, and writing—original draft preparation; Zaira Romeo was responsible for the methodology, data curation, investigation, and writing—review and editing; Lorenza Maistrello was responsible for the methodology and formal analysis; Eugenia Durgoni performed the data curation and investigation; Camilla Della Pietà performed the data curation and investigation; Michele De Filippo De Grazia performed the data curation and formal analysis; Francesca Meneghello as responsible for the conceptualization and project administration; Andrea Turolla was responsible for the conceptualization, methodology, writing–review and editing, supervision, and project administration; Marco Zorzi was responsible for the conceptualization, methodology, writing—review and editing, supervision, funding acquisition, and project administration.

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Supplementary Materials

In supplementary materials details of patients' demographic, clinical and experimental information (Table 1S-3S). Details of PCA (Figure 1S, Table 4S), correlation matrix (Table 5S, 6S), regression (Table 7S, 8S), and VLSM analyses (Table 8S-11S Figure 2S). *(Supplementary Materials)*

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Research Article

A New Neurorehabilitative Postsurgery Intervention for Facial Palsy Based on Smile Observation and Hand-Mouth Motor Synergies

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Objective. To perform a preliminary test of a new rehabilitation treatment (FIT-SAT), based on mirror mechanisms, for gracile muscles after smile surgery. *Method*. A pre- and postsurgery longitudinal design was adopted to study the efficacy of FIT-SAT. Four patients with bilateral facial nerve paralysis (Moebius syndrome) were included. They underwent two surgeries with free muscle transfers, one year apart from each other. The side of the face first operated on was rehabilitated with the traditional treatment, while the second side was rehabilitated with FIT-SAT. The FIT-SAT treatment includes video clips of an actor performing a unilateral or a bilateral smile to be imitated (FIT condition). In addition to this, while smiling, the participants close their hand in order to exploit the overlapped cortical motor representation of the hand and the mouth, which may facilitate the synergistic activity of the two effectors during the early phases of recruitment of the transplanted muscles (SAT). The treatment was also aimed at avoiding undesired movements such as teeth grinding. *Discussion*. Results support FIT-SAT as a viable alternative for smile rehabilitation after free muscle transfer. We propose that the treatment potentiates the effect of smile observation by activating the same neural structures responsible for the execution of the smile and therefore by facilitating its production. Closing of the hand induces cortical recruitment of hand motor neurons, recruiting the transplanted muscles, and reducing the risk of associating other unwanted movements such as teeth clenching to the smile movements.

1. Introduction

Moebius syndrome (MBS) is a rare neurological disorder characterized by bilateral nonprogressive congenital palsy of the facial (VII cranial) and abducens (VI cranial) nerves. Researchers estimate that the condition affects 1 in 50,000 to 1 in 500,000 newborns worldwide [1, 2]. In Italy, it is estimated that 5-6 individuals are born with MBS every year, yielding a total of about 500-600 affected patients [3]. Patients with MBS present facial and ocular symptoms at birth including reduced or absent facial expressiveness, incomplete eye closure, inability to perform lateral eye movements, and difficulty in sucking. Patients with MBS cannot perform movements such as closing their lips, pronouncing some language sounds, smiling symmetrically, closing their eyelids, or wiggling their eyebrows. They also present labial incompetence (i.e., drooling due to inability to effectively contain saliva) and difficulties in closing the eyelids, which may cause corneal ulcers or infections [4]. Other cranial nerves such as the glossopharyngeal and spinal accessory

may be involved, and patients may also present limb abnormalities (i.e., clubbed feet, congenital hand anomalies, and pectoral anomalies) in up to 15%-25% of cases [5]. Most importantly, the absence of facial mimicry hinders nonverbal communication, interfering greatly with social interactions and leading to psychological repercussions such as social stigma, marginalization, and depression [1, 6].

To date, the only available treatment to partially overcome facial palsy in MBS is surgical. Facial paralysis reconstruction (i.e., smile surgery) is aimed at achieving symmetry at rest and during dynamic facial movements, thus creating some degree of mobility in the lower face to produce facial expressions [7]. Depending on the origin of the facial palsy and on its evolution over time, patients may require a muscle transfer (free functional muscle transfer, FFMT) [3]. For patients with bilateral paralysis such as MBS, FFMT is the standard procedure aimed at restoring facial animation [8, 9] (for further details on FFMT, see supplementary online material). Rehabilitation requires a prolonged period after surgery, with the patient spending many months exercising facial movements under the guidance of a speech therapist [10]. At present, no consensus guidelines for the rehabilitative protocol are available for such forms of facial palsy. Nevertheless, once the muscle begins to show evidence of producing the first contractions, clinicians have found it effective to train patients to produce muscle contractions through a teeth clenching trigger under mirror feedback [11]. Although teeth clenching has proved effective in rapidly recruiting the transplanted muscles [12], clinicians also report difficulties in dissociating the movements of muscles for chewing from those of smiling. Therefore, long periods of rehabilitation are required before patients learn to move facial muscles independently and to dissociate the motor circuits involved in chewing and smiling [13]. Moreover, some patients report discomfort in observing their image reflected in a mirror, resulting in poor compliance during home training. Indeed, it is well known that facial palsy has negative consequences for self-perception [14, 15] due to facial asymmetry and absence of facial mimicry.

The purpose of the present study was to evaluate the feasibility of a new rehabilitation treatment after smile surgery. We propose a treatment based on action observation therapy (AOT) [16], which has been shown to have clinical and rehabilitative relevance [17–20], and which exploits the visuomotor coupling properties of the mirror neuron system (MNS) [21] as well as the motor synergies between the hand and the mouth present at a cortical level [22–26] to facilitate the recruitment of transplanted muscles in MBS patients.

1.1. Theoretical Assumptions of Facial Imitation Treatment (FIT). Mirror neurons were discovered in the ventral premotor region F5 of the macaque monkey more than twenty-five years ago [27, 28]. This class of neurons fires both when individuals execute a specific motor act and when they observe the same or a similar act performed by another individual [29–31]. The mirror mechanism is widely believed to support social cognitive functions such as action and emotion understanding by mapping perceived actions onto internal motor representations [32, 33]. Evidence suggests that mirror neurons are recruited in tasks requiring observation and imitation of actions and facial expressions [30, 33–36], empathy [37–40], and intentions [23, 41] and in language perception [42, 43]. These properties of mirror mechanisms can be exploited in neurorehabilitative treatments. For instance, in patients with motor deficits due to vascular brain injury or other neurological insults, the observation of a movement might improve movement recovery, reinforcing the activation of motor circuits which have been weakened due to the lesion [6, 17, 44]. This mechanism is the basis of AOT which combines exercises aimed at reducing the motor deficit with rehabilitation sessions whereby patients simultaneously observe the same exercises performed by the rehabilitator [16, 18].

In this study, we applied the principles underlying AOT to smile rehabilitation. According to embodiment theories [18, 30, 32, 43, 45], during the observation of emotional faces, affective and motor neural systems are activated together [1, 46–48] and people would react with congruent muscle activations (unconscious facial mimicry [49]) when looking at emotional facial expressions. This covert motor simulation of emotional faces [50] is supported by a broad network of regions with mirroring properties [49] that reflect an internal simulation of the perceived emotional expression. Consequently, perceiving another person displaying a facial expression would result in increased neural activity in the perceiver's motor, emotional, and somatosensory areas [49, 51]. Thus, we hypothesized that by observing an actor who is smiling, the neural circuits that control the smile in the MBS patient may facilitate the recruitment of the transplanted muscle (Figure 1 [21]).

1.2. Synergistic Activity Treatment (SAT): Theoretical Assumptions. The concept of synergy has been proposed to explain the functional modules that control hand shaping while an individual is grasping objects of different sizes. Classic somatotopic theories postulate that distinct clusters of neuronal populations are associated with specific hand muscles, fingers, or finger movements [52, 53] and that the organization of such movements is somatotopically organized in the motor cortex [22], which is known to be somatotopically organized in a set of subregions that control different segments of the body [52]. More recent views suggest that movements are represented in motor areas as clusters of neurons coding for different action types or goals [54]. For instance, Graziano and Aflalo [55] demonstrated that electrical stimulation of the rostral precentral gyrus evokes coordinated movements of the hand and mouth and that these movements seem to be present even within the restricted repertoire of behaviors of infant primates. In general, preset motor repertoires for ethologically relevant actions have been demonstrated in the monkey cortex by mapping studies with microstimulation of motor cortical areas [56]. These results are consistent with recent neuroanatomical studies of the human brain, which have shown that representations of the hand and mouth in the human motor cortex are contiguous and show a high degree of overlap [22]. This organization is generally believed to produce adaptive movements by optimizing neural resources associated to effectors that are jointly

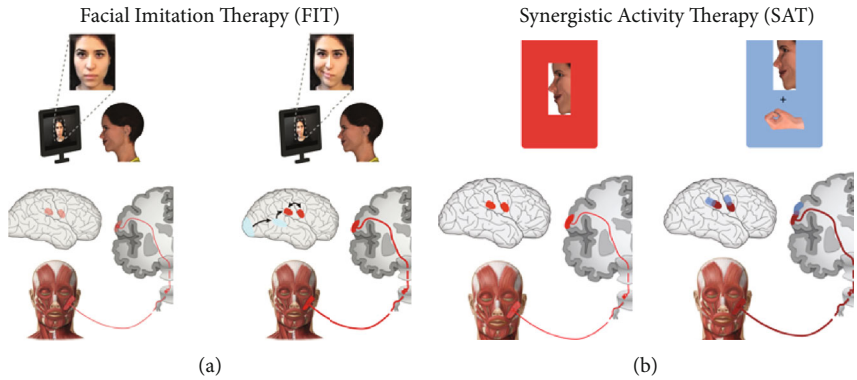

Figure 1: Modified from Ferrari et al. [21]: FIT-SAT theoretical assumptions. (a) FIT combined action observation with the direct effects of action execution suggesting that activation of motor areas by action observation becomes reinforced by the concomitant active execution of the observed actions[19]; (b) the synergistic activity of hand closing while smiling should facilitate the activation of the cortical areas connected to the mouth. We hypothesized that hand contraction would facilitate the recruitment of the gracilis muscle as a consequence of the activity of mouth motor neurons in motor cortical areas.

involved in coordinated actions. For instance, we often close our hands to grab edible objects with the aim of bringing food to the mouth. At the cortical level, the grasping movement and the mouth opening movement are represented as motor synergies for which the closure of the hand is accompanied by the opening of the mouth. These hand/mouth movements are synchronous and coordinated to maximize their efficacy. It has been demonstrated that during electrical stimulation of the sensorimotor cortex, the mouth starts to open while the closing hand moves towards the face [22]. Furthermore, numerous kinematics studies by Gentilucci and colleagues show that the movement of the hand during grasping simultaneously affects the kinematics of the mouth during different motor tasks [23, 25, 26]. As a consequence, we have assumed that the synergistic activity of hand closing while smiling should facilitate the activation of the cortical areas connected to the mouth, facilitating the recruitment of the gracilis muscle without grinding of the teeth (synergistic activity therapy, SAT, Figure 1 [21]).

1.3. FIT-SAT at Home. The FIT-SAT treatment includes videos containing instructions and daily exercises to be performed at home for up to six months (Figure 2(a)). The protocol is divided into two phases. The first (unilateral) phase is aimed at increasing muscle strength with unilateral exercises avoiding teeth grinding and begins when the patient starts to recruit the transplanted muscle. This phase consists of a series of video clips of an actor performing only unilateral smiles which are then imitated by the patient. Each video clip contains instructions concerning both the coactivation of the hand closed as a fist and the specific number of repetitions that the MBS patient must perform each day. The duration of the first phase varies from patient to patient depending on the muscle recruitment. The second phase of the treatment begins only after the patient is able to perform multiple repetitions of the unilateral movement maintaining the posture for at least three seconds. The second (bilateral) phase is aimed at synchronizing the contraction of both sides in order to obtain a harmonious movement and a natural smile. This is achieved by presenting clips of an actor smiling bilaterally and by giving instruction about the coactivation of the hands. Bilateral exercises include modulation tasks in which the patient is asked to perform maximum and small (gentle) smiles[8] in order to train and control the contraction force of the transplanted muscle/s.

One of the most complex aspects of home training is ensuring that patients perform the exercises correctly. To this aim, FIT-SAT's video clips start with instructions describing the exercises and during execution include auditory feedback in the form of an external voice that marks the timing of the observed smile to help the patient appreciate the rhythm of the smile to be performed. Thus, video clips help to sustain patient performance during home training. At each clinical assessment, patients are provided with clip materials according to their clinical status.

1.4. Assessing the Efficacy of FIT-SAT: Kinematic Acquisitions. The aim of the present study was to compare the efficacy of FIT-SAT with that of the traditional treatment. All patients underwent a two-stage surgery procedure (FFMT), spaced at least 9-12 months apart. They rehabilitated the right side of the face with traditional treatment [11, 15] first and about one year later the left side with FIT-SAT. We planned two kinematic acquisitions, one at the beginning of FIT-SAT (T1) and one at the end of treatment (T2, Figure 2(b)), to measure the three-dimensional motion of the patients' smile excursion. To compare the two treatments, we assessed maximal mouth aperture in the bilateral task between T1 and T2 to test how much the movement on one side of the face was the same as the movement on the other side. Specifically, we calculated the Euclidian distances between the left and right lip corner markers and the nose marker (Figure 3(b)). These parameters extrapolated from the bilateral smile provide an indirect measure of the left and right excursions, and their comparison may support the efficacy of the FIT-SAT treatment. Specifically, if the excursion of the left side at T2 was not different to that observed in the right side at T1, this would be evidence that FIT-SAT permitted a muscle recruitment as much as the traditional treatment [11]. Furthermore, we assess the efficacy of

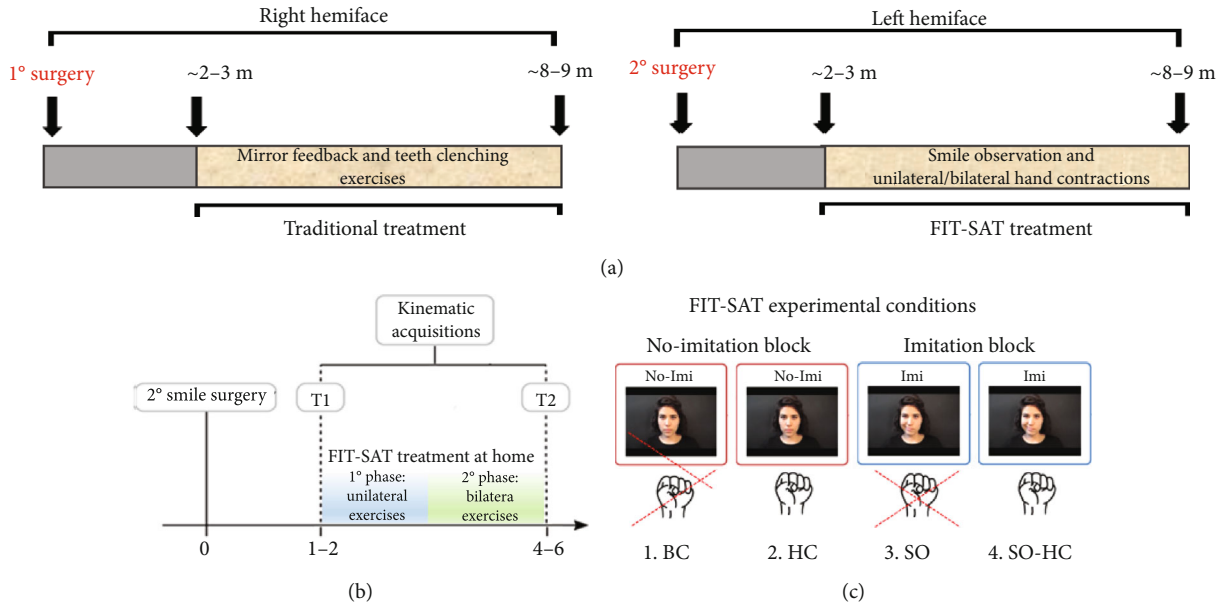

Figure 2: FIT-SAT treatment. (a) The FIT-SAT treatment was performed at home for about 6 months. After the first surgery, the right side of the face was rehabilitated by teeth clenching and mirror feedback. After the second surgery, the FIT-SAT treatment started as soon as the patient began to recruit the muscle. (b) The FIT-SAT treatment was divided into two phases: in the first (unilateral) phase, patients performed unilateral exercises in order to recruit the left transplanted muscle as soon as possible. The second (bilateral) phase started only after the patient was able to perform multiple repetitions of the unilateral left movement maintaining the posture for at least three seconds. From now on, the patient had to learn to coordinate the two sides of the face performing bilateral exercises. (c) Experimental condition: (1) no smile observation and no-hand contraction (baseline condition, BC), (2) no smile observation but hand contraction (HC), (3) smile observation but no-hand contraction (SO), and (4) smile observation and hand contraction (SO-HC).

FIT-SAT to improve left muscle recruitment at the beginning of the treatment and to reduce asymmetry at the end of the treatment.

2. Material and Methods

2.1. Design and Participants. A small sample, pre- and post-surgery experimental design was adopted to study the efficacy of FIT-SAT. Four bilateral patients with MBS were included. Each patient was surgically treated from 2016 to September 2018 (right and left sides of the face, respectively) at the maxillofacial surgical unit at the University of Parma Hospital. Inclusion criteria were (1) a certified diagnosis of congenital and bilateral facial paralysis; (2) a transplanted segment of the gracilis muscle in both sides of the face and the motor nerve to the masseter muscle used for innervation; (3) recruitment of the right gracilis muscle subject to traditional treatment using teeth clenching; (4) recruitment of the left gracilis muscle subject to FIT-SAT treatment; (5) absence of congenital hand malformations; (6) absence of any psychiatric or physical illness at the time of participation; (7) age greater than 6 years.

All participants first underwent an operation on the right side of the face. For the rehabilitation of the right transplanted muscle, they underwent traditional treatment with teeth clenching (Pavese et al., 2016). After about one year, participants underwent a second surgery on the left side of the face. The patients underwent FIT-SAT treatment [21] after this second surgery (Table 1). Consequently, the first operated side (the right one) can be considered a "control side" as it represents activation of the gracilis muscle using traditional treatment. Clinical practice did not allow us to randomize the side subjected to the FIT-SAT. This can represent a potential limitation as facial expressions are more intensely expressed in the left side of the face [57], and previous works found a main effect of sidedness of the face on aesthetic judgments of pleasantness with the left hemiface usually more expressive [58]. However, for the purposes of this study, we were evaluating only the excursion of the smile and its symmetry while further studies will be needed to evaluate the expressiveness of the face.

Written consent was obtained after full explanation of the research procedure, in agreement with the Declaration of Helsinki. The treatment was approved by the Joint Ethics Committee of the Parma Department of Medicine and Surgery and of the Parma Hospital on 12nd October 2016 (Prot. 34819).

2.2. Procedure. When the left transplanted muscle innervated by the masseteric nerve gave signs of activation (approximately 2-3 months after the second surgery), the patients started FIT-SAT treatment at home and underwent the first kinematic acquisition (T1). The second kinematic acquisition (T2) occurred at the end of FIT-SAT (about 8-9 months after the second surgery) to measure the patients' progress in recruiting the transplanted muscle (Figure 2(a)).

Kinematic data were obtained by means of an optoelectronic system for motion analysis (SMART-DX-100 system, BTS Bioengineering). This system consists of four digital infrared cameras (with a frequency of 100 Hz), which detect

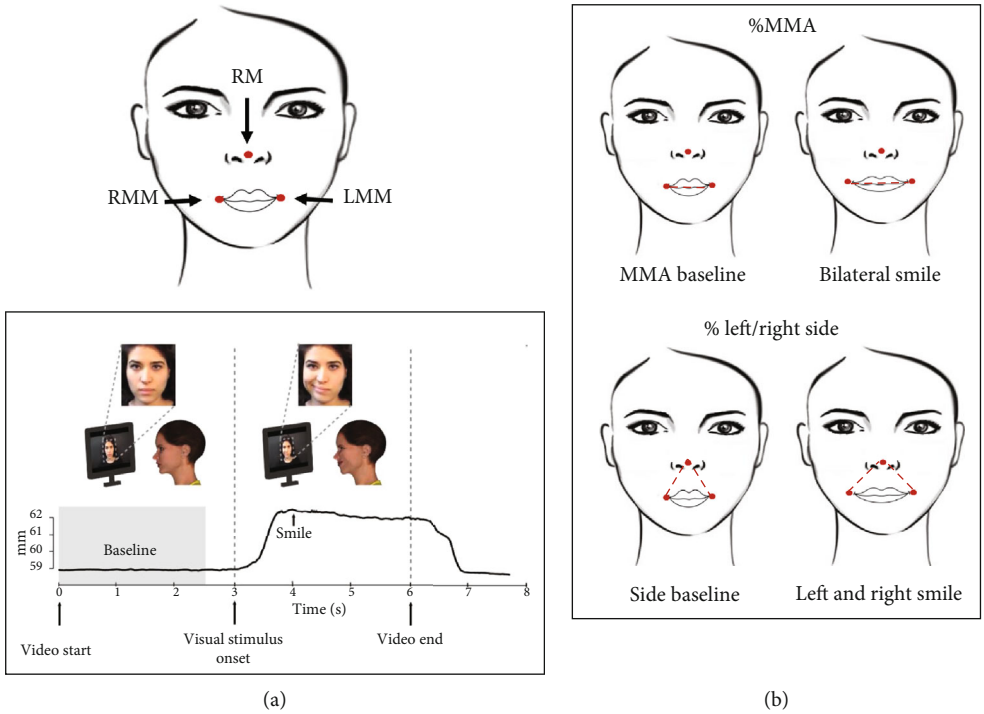

Figure 3: Kinematic parameters. (a) Example of one trial. The black line represents the excursion of the markers placed on the participant's mouth. The movement began after the participants observed the actress's smile and maintained the posture for about three seconds. The baseline is shown in gray. In this phase, the subject did not perform any movement. (b) Three reflective passive markers were placed on the participant's face (left mouth marker, LMM; right mouth marker, RMM; and reference marker, RM). Bilateral smile amplitude was calculated as the maximum Euclidian distance (MMA) in millimeters between the two lip corner markers (LMM and RMM). This measure was expressed as a percentage of the MMA at baseline (%MMA). Similarly, left/right side parameters were calculated as the Euclidian distances in millimeters between LMM or RMM lip corner marker and the nose marker (RM). Left/right side parameters were expressed as the percentage of side baseline (left or right, respectively, before movement onset).

Table 1: Patient classification: demographics and clinical characteristics of patients.

ID_ num	Sex	Age	Patients classification	Type of paralysis	Type of smile surgery	Transplanted muscle	1° smile surgery	2° smile surgery	FIT-SAT duration
MBS01	f	11	Bilateral Moebius	Complete bilateral paralysis	Free muscle transfer	Right side: gracile Left side: gracile	Right side 12-05-2015	Left side 21-01-2016	235
MBS02	f	40	Bilateral Moebius	Complete bilateral paralysis	Free muscle transfer	Right side: gracile Left side: gracile	Right side 03-02-2016	Left side 21-04-2017	205
MBS03	f	7	Bilateral Moebius	Complete bilateral paralysis	Free muscle transfer	Right side: gracile Left side: gracile	Right side 11-06-2016	Left side 31-08-2017	167
MBS04	m	8	Bilateral Moebius	Complete bilateral paralysis	Free muscle transfer	Right side: gracile Left side: gracile	Right side 01-07-2015	Left side 18-01-2017	147

the 3D movement of passive markers reflecting infrared rays emitted by illuminators with a spatial accuracy of at least 0.2 mm under the experimental conditions. Two markers were applied at the corners of the mouth (right and left mouth markers, RMM and LMM, respectively) and a further additional marker was placed on the nose (nose marker or reference point, RM, Figure 3(a)). Kinematic parameters were computed from each tracked trial using a custom program developed in RStudio 1.0.136 (https://www.rstudio.com/.).

Each kinematic acquisition consisted of 2 blocks: (1) imitation block in which an actress performed the smiles to be imitated by the patient; (2) no-imitation block in which an actress did not smile but provided the rhythm of the smiles during patients' assessment. Each block consisted of 40 repetitions of bilateral smiles and unilateral "half smiles." After FFMT, patients have active movement excursion bilaterally, but they are able to move each side of their mouth independently. Thus, we asked the participants to perform a left half smile (unilateral task) to measure mouth excursion in the

side rehabilitated with FIT-SAT. In both the imitation and no-imitation block, four experimental conditions were assessed:

(1) *Smile observation and hand/s contraction (SO-HC)*: patients first observed a video clip in which an actress executed unilateral or bilateral smiles and then smiled while simultaneously closing their ipsilateral hand or both hands.

(2) *Smile observation and no hand/s contraction (SO)*: patients observed/imitated unilateral or bilateral smiles maintaining their hand/s relaxed in a prone position

(3) *No smile-observation and hand contraction (HC)*: the actor was visible on the screen and provided auditory feedback that marked the timing of the patients' smiles. Following the instructions of the actress on the video, the patients performed unilateral or bilateral smiles while simultaneously closing their ipsilateral hand or both hands.

(4) *No smile observation and no hand contraction (BC)*: patients simply performed unilateral or bilateral smiles. We refer to this condition as the baseline condition (Figure 2(b)).

Patients performed 40 left and 40 bilateral smiles (10 repetitions for each experimental condition), 80 smiles in total. Each video lasted six seconds, three seconds of instruction followed by three seconds for performing the exercise (Figure 2(b)). Between each trial, patients could pause if they so desired. The order of the blocks was randomized among subjects.

2.3. Kinematic Parameters. Bilateral smile amplitude was calculated as the maximum Euclidian distance (MMA) in millimeters between the two lip corner markers (Figure 3(b)). This measure was expressed as a percentage of the MMA at baseline (%MMA), the MMA baseline corresponding to the Euclidian distance between the lip corner markers before movement onset (0 to 2.5 seconds, Figure 3(b)). For all trials, the %MMA was therefore calculated as follows:

$$\%MMA = \frac{MMA - MMA\ baseline}{MMA\ baseline} * 100. \quad (1)$$

In unilateral blocks (unilateral task), left %MMA was the Euclidian distance in millimeters between the two lip corner markers expressed as a percentage of the MMA at baseline.

Left (or right) smile excursions (left/right side) were also calculated as the Euclidian distances in millimeters between the left (right) lip corner marker and the nose marker (Figure 3(b)). Left/right side parameters were expressed as the percentage of side (% left/right side) with respect to the left/right side baseline (the Euclidian distance between the lip corner markers measured before movement onset (0 to 2.5 seconds, Figure 3(a))). For all trials, % left/right side was therefore calculated as follows:

$$\%Left\ side = \frac{Left\ side - Left\ side\ baseline}{Left\ side\ baseline} * 100,$$
$$\%Right\ side = \frac{Right\ side - Right\ side\ baseline}{Right\ side\ baseline} * 100. \quad (2)$$

We also calculated the asymmetry index of the bilateral blocks (bilateral task) (%AI), which provides information to evaluate the attainment of a harmonious and natural movement. The AI was calculated with the following formula:

$$\%AI = \left(\frac{\max^{-}MMA - \min^{-}MMA}{\max^{-}MMA + \min^{-}MMA}\right) * 100. \quad (3)$$

A smile will be symmetrical as the value approaches 0% asymmetric as the value tends to 100% [59].

2.4. Statistical Analysis. The aim of this study was to compare the efficacy of standard treatment with FIT-SAT. Right and left sides of the face were operated in two phases (about one year apart). As a result, one side was rehabilitated before the other. All patients rehabilitated the right side of the face with traditional treatment [11] first and about one year later the left side with FIT-SAT. The main objectives were the following:

(1) to assess the excursion of the left half smile (Left %MMA) among experimental conditions at T1

(2) to assess an improvement in symmetry (%AI reduction) between T1 and T2

(3) to compare participants' maximal mouth aperture between % right side at T1 and % left side at T2

We used linear mixed-effect models fit by maximum likelihood (LMM) to test the efficacy of the FIT-SAT treatment on the rehabilitation of the patients' smile. To select the best model that yields our data, we used the Akaike information criterion (AIC), which offers a principled balance between goodness-of-fit and model complexity [60]. The principal characteristic of this approach is the inclusion of random subject effects into regression models in order to account for the influence of subjects on their repeated observations. The information criteria (AIC values) together with log-likelihood statistics are reported and provide a way to assess the fit of a model based on its optimum log-likelihood value (Tables 2–4). Data analyses were performed using RStudio 1.3.1093 (https://www.rstudio.com/.) using the "Ime" function in the "nlme" package. The threshold for statistical significance was set at $p < 0.05$ for all analyses.

3. Results

3.1. Unilateral Task. To test the FIT-SAT conditions in facilitating the unilateral left excursion (first phase) at the beginning of the treatment, we entered left %MMA as the

Table 2: FIT-SAT treatment efficiency: best fit mixed-effect models (unilateral smile in T1).

Parameters	Model	df	AIC	BIC	LogLik	Test	L. ratio	p value
Left %MMA	$m0_{\_T1}$	2	700.4	706.5	-348.2			
	$m1_{\_T1}$	3	589.9	598.9	-291.9	$m0_{\_T1}$ vs. $m1_{\_T1}$	112.6	<0.001
	$m2_{\_T1}$	4	581.3	599.4	-284.8	$m1_{\_T1}$ vs. $m2_{\_T1}$	14.5	<0.002

Table 3: FIT-SAT treatment efficacy: best fit mixed-effect models. Information of the mixed-effect models used for different kinematic parameters.

Parameters	Model	df	AIC	BIC	LogLik	Test	L. ratio	p value
%MMA	m0	2	1445.9	1453.2	-721.0			
	m1	3	1285.4	1296.3	-639.7	m0 vs. m1	162.5	<0.001
	m2	4	1234.9	1249.4	-613.4	m1 vs. m2	52.5	<0.001
	m3	7	1230.4	1255.8	-608.2	m2 vs. m3	10.5	0.015
	m4	10	1229.3	1265.5	-604.6	m3 vs. m4	7.2	0.066
%AI	m0	2	1595.7	1602.9	-795.9			
	m1	3	1509.1	1519.8	-751.5	m0 vs. m1	88.7	<0.001
	m2	4	1402.9	1417.2	-697.5	m1 vs. m2	108.2	<0.001
	m3	7	1408.0	1432.9	-697.0	m2 vs. m3	1.0	0.813
	m4	10	1413.2	1448.9	-696.6	m3 vs. m4	0.8	0.859
% left side	m0	2	1337.5	1344.7	-666.7			
	m1	3	1267.1	1277.9	-630.5	m0 vs. m1	72.4	<0.001
	m2	4	1121.8	1136.3	-556.9	m1 vs. m2	147.2	<0.001
	m3	7	1125.9	1151.3	-556.0	m2 vs. m3	1.9	0.598
	m4	10	1130.8	1167.0	-555.4	m3 vs. m4	1.2	0.764
% right side	m0	2	1331.0	1338.2	-663.5			
	m1	3	1123.3	1134.2	-558.7	m0 vs. m1	209.7	<0.001
	m2	4	1093.2	1107.7	-542.6	m1 vs. m2	32.2	<0.001
	m3	7	1091.4	1116.7	-538.7	m2 vs. m3	7.8	0.050
	m4	10	1093.1	1129.4	-536.6	m3 vs. m4	4.2	0.238

Table 4: FIT-SAT treatment efficiency: best fit mixed-effect models (bilateral smile, % left vs. right side).

Parameters	Model	df	AIC	BIC	LogLik	Test	L. ratio	p value
% left/right side	m0	2	2678.1	2686.7	-1337.0			
	m1	3	2440.9	2453.8	-1217.4	m0 vs. m1	239.2	<0.0001
	m2	4	2418.3	2435.6	-1205.2	m1 vs. m2	24.6	<0.0001
	m3	7	2397.6	2419.2	-1193.8	m2 vs. m3	22.7	<0.0001
	m4	10	2238.3	2264.2	-1113.1	m3 vs. m4	161.3	<0.0001

dependent variable and compared the fit of a generalized least squares (GLS) null model ($m0_{\_T1}$, $y \sim 1$) with fixed intercept with that of a null model with random intercept ($m1_{\_T1}$, $y \sim (1\,\text{subjects})$). $m1_{\_T1}$ provided a superior fit than $m0_{\_T1}$ ($AIC_{m0\_T1} = 700.4$ and $AIC_{m1\_T1} = 589.9$; $p < 0.001$). We then added the factor "condition" as a fixed effect to m1, generating $m2_{\_T1}$ ($y \sim \text{condition} + (1\,\text{subjects})$). The comparison between models revealed that $m2_{\_T1}$ provided a better fit ($AIC_{m2\_T1} = 581.3$; $p < 0.002$, see Table 2).

Post hoc tests (Dunnett's) were performed to test the condition effects. We observed a significant increase in SO-HC (5.55 mm ± 0.4) in comparison to BC (4.47 mm ± 0.4, $p = 0.005$, Figure 4). No other comparisons were found to be significant ($p > 0.05$).

3.2. Bilateral Task. On average, %MMA increased at the end of FIT-SAT treatment (T2) with respect to the beginning T1 (T1 = 12.59 mm ± 0.19, T2 = 14.66 mm ± 0.32; Figure 5(a)) whereas %AI decreased (T1 = 10.45 mm ± 0.52, T2 = 5.59 mm ± 0.24; Figure 5(b)). Similar to %MMA, in T2, the % left side increased in the percentage of excursion in comparison to T1 (T1 = 0.616 mm ± 0.11, T2 = 3.593 mm ± 0.24; Figure 5(c)) whereas the average values of the % right side

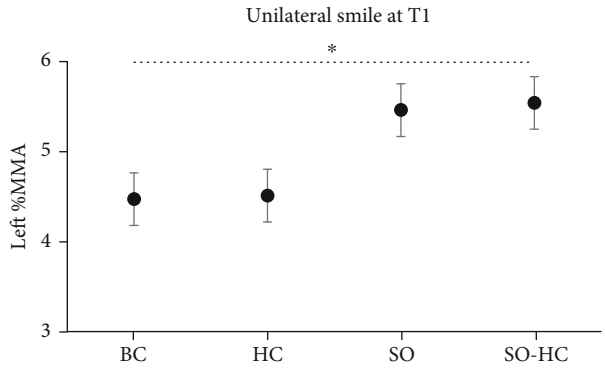

Figure 4: Results of unilateral task at T1. Left %MMA was the Euclidian distance in millimeters between the two lip corner markers expressed as a percentage of the MMA at baseline. All the experimental conditions are represented: smile observation followed by imitation of the same smile movement and ipsilateral hand contraction (SO-HC), smile observation followed by imitation of the same smile movement but without hand contraction (SO), no smile observation but hand contraction (HC), and no smile observation and no hand contraction (BC). Error bars represent SE (standard errors of the means).

show a slight decrease (T1 = 3.544 mm ± 0.28, T2 = 2.39 mm ± 0.15; Figure 5(d)).

We run LMM with a random intercept to account for the interindividual variability, and we compared models using the likelihood-ratio test. We entered all kinematic parameters as the dependent variables and compared the fit of a generalized least squares (GLS) null model (m0, $y \sim 1$) with fixed intercept with that of a null model with random intercept (m1, $y \sim (1 \text{ subjects})$). In %MMA, m1 provided a superior fit than m0 ($AIC_{m0} = 1445.9$ and $AIC_{m1} = 1285.4$; $p < 0.001$). We then added the factor "acquisition" as a fixed effect to m1, generating model 2 (m2, $y \sim \text{acquisition} + (1 \text{ subjects})$). The comparison between models revealed that m2 provided an even better fit ($AIC_{m2} = 1234.8$; $p < 0.001$), suggesting that mouth maximal aperture increased as a function of time. Finally, we added the factor "condition" as a fixed factor (m3, $y \sim \text{acquisition} + \text{condition} + (1 \text{ subjects})$) and interaction (m4, $y \sim \text{acquisition} * \text{condition} + (1 \text{ subjects})$). The comparison between models revealed that m3 provided the better fit ($AIC_{m3} = 1230.4$; $p < 0.015$, see Table 3). Dunnett's comparisons were performed comparing each FIT-SAT condition (HC, SO, and SO-HC) with the control condition (BC). We observed a significant increase in SO-HC condition (14.19 mm ± 0.37) in comparison to BC (13.36 mm ± 0.39, $p = 0.045$, Figure 6). No other differences were found ($p > 0.05$).

We performed the same comparisons between models in the %AI variable. We observed a lower AIC values in both m0 vs. m1 and m1 vs. m2 comparisons ($AIC_{m0} = 1595.7$ and $AIC_{m1} = 1509$, $p < 0.001$; $AIC_{m2} = 1402.9$, $p < 0.001$; Table 3). Specifically, the factor "acquisition" improves the quality of the fit compared to m0 and m1 suggesting that, in T2 patients, smiles were more symmetrical than in T1 patients. Thus, the best explanation for the improvement in the quality of patients' smile was accounted by the factor "acquisition" which, in turn, reflects the effect of the FIT-SAT treatment over time (Figure 5(b)). Instead, model comparisons indicated that m3 and m4 did not improve the fitting ($p > 0.05$, Table 3).

To analyze the effect of the FIT-SAT treatment in activating the left muscle without teeth clenching, we further employed a LLM for left and right sides separately. Once again, the best model that yields our data in the excursion of % left side was accounted for by the acquisition factor ($AIC_{m2} = 1121.8$; $p < 0.001$, Figures 5(c) and 5(d)). The AIC values for each comparison between models are shown in Table 3.

3.3. Traditional vs. FIT-SAT Treatment Comparison. To examine the two treatments, we compared the % left side and % right side parameters at T1 and T2. The analysis procedure follows the previous one. The comparison between models revealed that m4 provided the better fit (AICm4 = 2238.3; $p = 0.001$, see Table 4).

Dunnett's comparisons were performed comparing % right side T1 with the other conditions. We found a significant difference between % right side T1 and % left side T1 (3.54 ± 0.28 and 0.62 ± 0.11, respectively; $p < 0.001$, Figure 7) and % right side T2 (1.17 ± 0.21, $p < 0.001$). No difference was found between % right side T1 and % left side T2 ($p > 0.05$).

4. Discussion

Peripheral facial palsy, involving a lesion of cranial nerves involved in facial mimicry, is typically correlated to important functional and aesthetic deficits. Patients with congenital unilateral or bilateral facial palsy show reduced or absent expressivity; they either cannot smile (when affected bilaterally) or find it very difficult to smile (unilateral paralysis). In addition, they cannot grimace or close their eyes normally. Finally, because of the lack of strength in their lip muscles, they also have problems with chewing, swallowing, and speaking. Surgical interventions are aimed at reducing the symptoms and restoring a degree of facial mobility (i.e., facial reanimation [7]). Despite the strong negative impact of facial palsy on psychosocial functioning and quality of life [61], however, current approaches to postsurgery treatment remain largely unsatisfactory. Following muscle transplant, traditional rehabilitation programs are aimed at activating newly formed motor circuits under the control of the masseteric nerve. Thus, patients are initially encouraged to practice biting in front of a mirror [11]. However, the practice of teeth clenching, although extremely effective in recruiting the transplanted muscles [12], leads to difficulties in separating chewing from smiling and remains divorced from mimicry processes, which play an important part in social interactions. As an additional problem, clinicians report poor compliance with prescriptions involving home training under mirror feedback, presumably due to the negative consequences of facial palsy for self-perception [14].

Here, we tested a new neurorehabilitative protocol (FIT-SAT) that exploits the properties of the mirror system as well as hand-mouth synergies [22, 55] related to the somatotopic

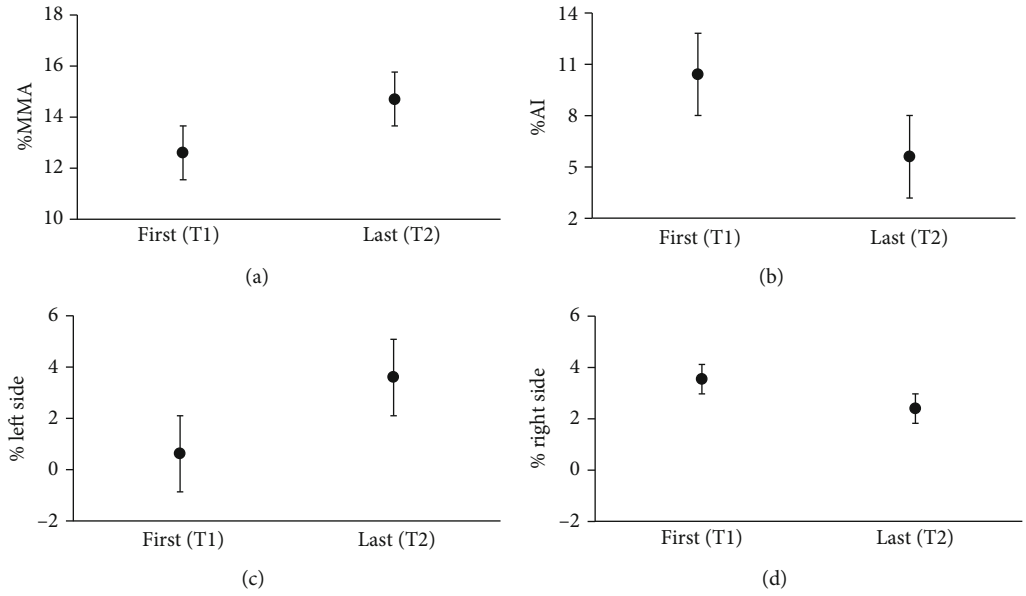

Figure 5: The graphs show the results of the bilateral analysis between the first (T1) and the last acquisition (T2). The parameters considered were (a) %MMA (the maximum Euclidian distance in millimeters between the two lip corner markers), (b) %AI (asymmetry index), (c) % left side (the Euclidian distances in millimeters between the left lip corner marker and the nose marker), and (d) right side (the Euclidian distances in millimeters between the right lip corner marker and the nose marker). Error bars represent SE (standard errors of the means).

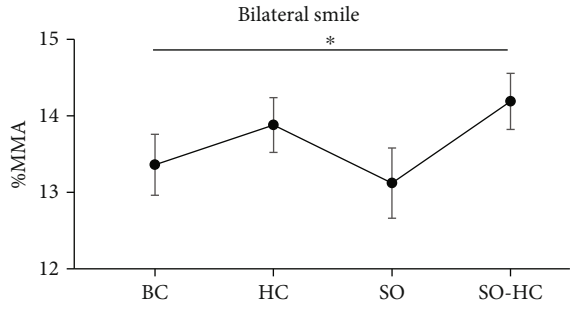

Figure 6: The graph shows the results of the bilateral task in both acquisitions considering the FIT-SAT conditions. Specifically, %MMA (the maximum Euclidian distance in millimeters between the two lip corner markers) increased in SO-HC (smile observation and hand contraction) with respect to the baseline (BC). Error bars represent SE (standard errors of the means).

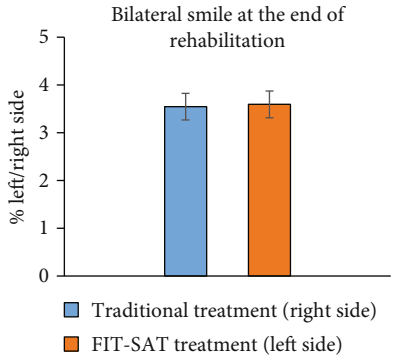

Figure 7: The graph shows the results between the % right side and the % left side (the Euclidian distances in millimeters between the left/right lip corner marker and the nose marker, Figure 3(b)) at the first acquisition (T1, blue) and at the last acquisition (T2, orange), respectively. Error bars represent SE (standard errors of the means).

organization of the motor cortex. Our results support the feasibility of FIT-SAT as an alternative to mirror feedback therapy. Specifically, we analyzed the excursion of the lips in four patients with bilateral paralysis. The patients rehabilitated the right side of the face with the traditional treatment involving teeth clenching [11], whereas they rehabilitated the left side with FIT-SAT [21]. Using 3D kinematic acquisitions, the recruitment of the left transplanted muscle was monitored by the second intervention onwards. A beneficial effect of the SO-HC condition was observed in the unilateral task at the first acquisition. Specifically, smile observation (SO) associated to hand contraction (HC) was effective in recruiting the transplanted muscle in the early phase of the treatment (unilateral phase) resulting in a greater left side excursion with respect to the baseline (BC).

The unilateral phase of the FIT-SAT treatment finished when patients were able to recruit the transplanted muscle even in the absence of hand contraction. Once the first unilateral phase was completed and the muscle of the left side had been fully recruited, the second bilateral phase began. This second phase was aimed at synchronizing the contraction of both sides in order to obtain a harmonious movement and a natural smile. The most important result observed in the bilateral task was the improvement in smile symmetry at the end of the treatment.

In the bilateral task, we also observed a condition effect. Specifically, results showed an increase in the maximal mouth aperture in SO-HC in comparison to BC suggesting that the hand (effective in early muscle recruitment) was still useful at the end of the treatment by increasing lip excursion

during smiling when associated with smile observation. Nevertheless, it should be noted that the maximal mouth aperture is not the parameter that can best describe an improvement in the smile quality, and a greater maximal mouth aperture does not necessarily imply that the patient achieved a more harmonious and natural smile. As an example, an excessive excursion might rather indicate poor quality of modulatory control of muscles.

Finally, in the bilateral task, we did not find significant differences comparing the excursion of the right side at T1 (side of the face rehabilitated with traditional treatment) and the left side at T2 (side of the face rehabilitated with FIT-SAT treatment). This last result supports the conclusion that FIT-SAT treatment may be as effective as the traditional treatment in recruiting muscles involved in smiling after smile surgery. Notably, we found a significant decrease in right side excursion between T1 and T2. This effect could depend on FIT-SAT treatment. In fact, in the second phase of the FIT-SAT, bilateral exercises of modulation were included. This may have resulted in better smile control making the subject aware of the force of muscle contraction. These results, although promising, will require further investigations; in particular, it will be interesting to verify the modulatory effects of the FIT-SAT treatment over time.

One of the foremost goals for MBS patients undergoing postsurgical rehabilitation is to achieve a smile that is as harmonious and natural as possible. Our results indicate that FIT-SAT may be helpful in this respect as well, as we observed that smile symmetry improved between the first and last acquisitions. Thus, the combined use of smile observation, smile reproduction, and contingent hand contraction resulted in a reduction of the anomalous asymmetry.

A final consideration is in order in relation to the social function of smiling. The absence of a spontaneous smile is what brings most problems to patients suffering from facial paralysis since it impairs communication and social interaction [62]. In these patients, smile production cannot be controlled by a sensitive nerve, which means that they must control the smile consciously. Nevertheless, some authors have reported that, over time, some MBS patients develop an ability to activate their smile in social situations, especially if they underwent smile surgery at an early age [63, 64]. These reports have been used to propose that greater brain plasticity in younger patients leads to the achievement of a spontaneous smile after neural reorganization of involved motor processes [63, 64]. We speculate that FIT-SAT could favor this process. The motor and premotor cortexes have been demonstrated to be part of a visuomotor coupling mechanism (i.e., the mirror neuron system [65]). During the observation of an action/gesture, our motor system resonates with that of the model because the observer is automatically recruiting the same motor programs of the model. Motor resonance mediated by the above-mentioned sensorimotor mirror system could support basic functions such as action perception, understanding, and imitation of the observed agent [66], including mimicry which normally occurs during face-to-face interactions [67].

Here, both SO and SO-HC conditions exploit the principles of AO [16] to facilitate the recruitment of the transplanted muscle. Specifically, two mechanisms intervene: one is linked to the voluntary production of the smile, involving motor areas that provide awareness to the movement; the other one is based on activities of the MNS, an observation-execution matching system activated both during the execution of a motor act and during the passive observation of other people performing the same movement [29, 39]. In other terms, we map what we observe onto our own neural motor representations for a specific action, sensation, or emotion [1, 47, 48]. In fact, MNS is thought to crucially subserve emotion recognition processes. Not by chance, the temporary reversible lesion of the MNS (due to repetitive transcranial magnetic stimulation) is associated with performance deficits on tasks requiring the recognition of facial expressions of emotion [68]. To date, how voluntary and automatic processes interact is not entirely clear. Investigations conducted by Caruana et al. [69] by means of electrical stimulation during brain surgery supported the role of frontal operculum (FO) in both observation and the voluntary control of facial expressions. Its stimulation in patients that underwent brain surgery induced the production of a smile. Moreover, previous brain imaging studies have reported the activation of the FO both during the voluntary imitation and during the passive observation of a smile [70–72]. Thus, thanks to its connectivity pattern with other brain structures involved in emotion processing, FO would result in a sort of "gate" between the voluntary motor system and the emotional network and crucially subserving facial expression production and recognition in the context of social interactions. Thus, FIT-SAT may improve not only the recovery of motor function but also the spontaneity of the smile normally occurring in everyday social situations. In fact, when the patient smiles at another person who responds with eye contact [73, 74] and by smiling back, there is a powerful reinforcement both consciously and unconsciously, which likely aids the learning process as the patient can realize that the movement was indeed recognized as a smile. Such a speculation is supported by studies on mother-infant interactions, showing that infants tend to increase social expressiveness when their mothers mirror their facial expressions [75]. Moreover, such mother mirroring has an impact on the development of cortical motor circuits involved in facial expression perception [76]. However, at the moment, we have no actual evidence of the efficacy of FIT-SAT treatment in the production of a spontaneous smile, and future follow-up studies are needed to investigate the validity of this hypothesis.

4.1. Limitations of the Study. Because of the rarity of the syndrome, we could only include a small number of participants, and this precludes generalization of our results. For future studies, the research question should be addressed in a larger sample. For reasons related to clinical practice, it was not possible to randomize the side of the face rehabilitated with the FIT-SAT. Future studies will need to consider this aspect in order to obviate possible effects caused by hemispheric lateralization in emotion processing [58].

5. Conclusion

Our results indicate that hand contraction and smile observation may be as efficacious as traditional teeth clenching treatment, while bypassing patients' difficulties in working with the mirror and allowing a correct dissociation between chewing and smiling. To the best of our knowledge, this study is the first to apply an AOT-based rehabilitation approach [17, 18, 77] to patients with facial paralysis who undergo smile surgery [7, 78] and to integrate knowledge derived from neuroscience such as hand-mouth synergy with the clinical rehabilitation needs of these patients [22, 23, 43, 54]. Although this preliminary data is encouraging, further confirmation will be necessary with a greater number of patients and with experimental designs including assessments of FIT-SAT after the first muscle transplant.

Abbreviations

MBS: Moebius syndrome
FFMT: Free functional muscle transfer
FIT: Facial imitation treatment
SAT: Synergistic activity therapy
AOT: Action observation therapy.

Data Availability

Data are available upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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Supplementary Materials

Free functional muscle transfer (FFMT) description. *(Supplementary Materials)*

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Research Article

Distinction of High- and Low-Frequency Repetitive Transcranial Magnetic Stimulation on the Functional Reorganization of the Motor Network in Stroke Patients

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Objective. To investigate the functional reorganization of the motor network after repetitive transcranial magnetic stimulation (rTMS) in stroke patients with motor dysfunction and the distinction between high-frequency rTMS (HF-rTMS) and low-frequency rTMS (LF-rTMS). *Methods*. Thirty-three subcortical stroke patients were enrolled and assigned to the HF-rTMS group, LF-rTMS group, and sham group. Each patient of rTMS groups received either 10.0 Hz rTMS over the ipsilesional primary motor cortex (M1) or 1.0 Hz rTMS over the contralesional M1 for 10 consecutive days. A resting-state functional magnetic resonance imaging (fMRI) scan and neurological examinations were performed at baseline and after rTMS. The motor network and functional connectivities intramotor network with the core brain regions including the bilateral M1, premotor area (PMA), and supplementary motor area (SMA) were calculated. Comparisons of functional connectivities and Pearson correlation analysis between functional connectivity changes and behavioral improvement were calculated. *Results*. Significant motor improvement was found after rTMS in all groups which was larger in two rTMS groups than in the sham group. The functional connectivities of the motor network were significantly increased in bilateral M1, SMA, and contralesional PMA after real rTMS. These changes were only detected in the regions of the ipsilesional hemisphere in the HF-rTMS group and in the regions of the contralesional hemisphere in the LF-rTMS group. Significantly changed functional connectivities of the intramotor network were found between the ipsilesional M1 and SMA and contralesional PMA, between contralesional M1 and contralesional SMA, between contralesional SMA and ipsilesional SMA and contralesional PMA in the HF-rTMS group in which the changed connectivity between ipsilesional M1 and contralesional PMA was obviously correlated with the motor improvement. In addition, the functional connectivity of the intramotor network between ipsilesional M1 and contralesional PMA was significantly higher in the HF-rTMS group than in the LF-rTMS group. *Conclusion*. Both HF-rTMS and LF-rTMS have a positive effect on motor recovery in patients with subcortical stroke and could promote the reorganization of the motor network. HF-rTMS may contribute more to the functional connectivity reorganization of the ipsilesional motor network and realize greater benefit to the motor recovery.

1. Introduction

Interhemispheric imbalance and reduced interactions of neural activity and functional connectivity have been reported in both animal and human studies after stroke with motor dysfunction [1–4]. In addition, as the level of impairment increased, the network balance was more disrupted [5]. Therefore, the balance of the motor network between the two brain hemispheres is crucial for functional motor recovery of stroke patients [6]. Noninvasive brain stimulation, e.g.,

repetitive transcranial magnetic stimulation (rTMS), has been recognized as an effective strategy to facilitate motor recovery by enhancing/suppressing neural excitability of ipsilesional/contralesional hemispheres to restore interhemispheric balance [7–9]. Finally, these lead to cerebral plasticity and reorganization of the motor network of the damaged hemisphere.

Numerous functional neuroimaging studies have confirmed that recovery of motor function after stroke is commonly attributed to cortical reorganization of both ipsilesional sensorimotor areas and contralesional motor areas [10–13]. This reorganization is adaptive and is gradually shifted during the process of regaining motor function in the affected limbs. Additionally, reorganization of the ipsilesional hemisphere is traditionally believed to be most important for successful recovery [14]. Findings from a study of low-frequency rTMS (LF-rTMS) over the contralesional primary motor cortex (M1) suggested that one single session of rTMS could transiently remodel the architecture of the disturbed motor network, reflected as reduced transcallosal influences and a restitution of ipsilesional functional connectivity, in particular, the effective connectivity between M1 and supplementary motor area (SMA) [15]. Another stroke study with long-term high-frequency rTMS (HF-rTMS) treatment observed increased interhemispheric functional connectivity between ipsilesional M1 and contralesional motor areas [16]. Dual-mode stimulation combined with transcranial direct current stimulation (tDCS) also detected noticeably increased interhemispheric connectivity in subacute stroke patients [17]. However, in these studies, the difference between HF-rTMS and LF-rTMS on the influence of functional reorganization of the motor network was still not clear. The relationship between motor network reorganization and motor improvement has not been clarified. Maybe the restoration of some part of the motor network showed greater contribution to the recovery of motor function than others.

Therefore, to further clarify the reorganization of interhemispheric and intrahemispheric functional connectivity of the motor network and the relationship with motor recovery of rTMS, this study was aimed at investigating the connectivity changes between brain regions of the motor network after HF-rTMS or LF-rTMS. The comparison of the motor network changes after HF-rTMS and LF-rTMS was also conducted to ascertain their different modulation mechanisms on the motor network. We hypothesized that significantly increased functional connectivities and their correlation with motor improvement would be observed in some motor areas after HF-rTMS or LF-rTMS. The influence on the motor network may be distinct between them.

2. Materials and Methods

2.1. Participants. Thirty-three right-handed stroke patients (mean age: 64.48, range 53-78 years) with motor deficits after a first-onset subcortical ischemic stroke in the territory of the left middle cerebral artery were enrolled from the Department of Neurology at the Second Clinical Medical College of North Sichuan Medical College (Nanchong, China) according to the following inclusion criteria: (1) right handedness, (2) ischemic lesion at the unilateral subcortical area confirmed by diffusion-weighted imaging (DWI), (3) showing unilateral motor dysfunction, (4) no history of neurological/psychiatric diseases, and (5) no contraindications of rTMS and MRI measurement. Exclusion criteria were as follows: (1) hemorrhagic stroke, (2) any other brain disorder or abnormalities, (3) history of drug dependency or psychiatric disorders, (4) severe white matter hyperintensity, (5) substantial head movement during the fMRI data acquisition according to the preprocessing result, and (6) contraindication to MRI and/or TMS.

According to the Helsinki Declaration, this study was approved by the Ethics Committee of the Second Clinical Medical College of North Sichuan Medical College. This study was registered in the Chinese Clinical Trial Registry (ChiCTR-IOR-16008629) and reported following the guidelines of the Consolidated Standards of Reporting Trials (CONSORT) group. All participants gave informed consent before the experiment.

2.2. Study Design. All stroke patients were enrolled at the acute stage with a subcortical lesion location encompassing the left internal capsule, basal ganglia, or corona radiate. These patients were assigned to the HF-rTMS group (11 subjects, five males and six females, mean age 65.09 ± 5.84, range 58-75 years), LF-rTMS group (12 subjects, five males and seven females, mean age 63.58 ± 7.95, range 53-78 years), and sham group (10 subjects, five males and five females, mean age 64.90 ± 6.23, range 58-75 years). Each patient received rTMS daily for 10 consecutive days. An MRI scan and several comprehensive neurological examinations including the National Institutes of Health Stroke Scale (NIHSS), Fugl-Meyer Assessment (FMA), and Barthel Index (BI) were performed prior to the experiment and immediately after 10 days of rTMS. Based on these scales, the stroke severity, motor impairment, and daily living ability were evaluated.

2.3. Intervention. After stroke, the equilibrium of cortical excitability between the two hemispheres is disrupted. This has shown decreased excitability of the ipsilesional hemisphere and increased excitability of the contralesional hemisphere [18]. Based on the interhemispheric competition model, previous studies have reported that the inhibitory rTMS on the contralesional hemisphere could increase excitability of the ipsilesional motor cortex by reducing excessive interhemispheric inhibition from the contralesional motor cortex [19, 20], whereas excitatory rTMS over the affected hemisphere directly increases the excitability of the ipsilesional motor cortex [21, 22]. Therefore, the strategy of HF-rTMS over the ipsilesional motor cortex and LF-rTMS over the contralesional motor cortex was selected in our study.

rTMS was performed by using a Magpro R30 stimulator (MagVenture, Lucernemarken, Denmark) equipped with a 70.0 mm butterfly-shape coil and a handle posterior and oriented sagittally. The scalp site that could elicit response in the first dorsal interosseous muscle of the affected/unaffected hand was selected as the optimal location of the center of

the rTMS coil for HF-rTMS/LF-rTMS intervention. If nonresponsive activity could be detected stimulating the ipsilesional M1 for the patients in the HF-rTMS group, symmetric location homologous to the contralesional M1 would be defined as the stimulation site. A resting motor threshold (RMT) was established and was defined as the lowest rTMS intensity that could elicit a motor-evoked potential of at least an amplitude of 50 ?V in at least half of 10 consecutive stimuli over the M1 [23]. Stimulation was applied at 90% RMT at 1.0 Hz frequency (900 pulses) over contralesional M1 in the LF-rTMS group (30 trains, 30 pulses/train, intertrain interval = one second, and a total of 900 pulses) and at 90% RMT at 10.0 Hz frequency (30 trains, 50 pulses/train, intertrain interval = 25 seconds, and a total of 1,500 pulses) over ipsilesional M1 in the HF-rTMS group. The sham group received rTMS with the same parameters as the LF-rTMS group over the contralesional M1 but without real stimulation to ensure that no current flow was induced in the brain. All rTMS sessions were performed in the same room. All stroke patients received the same physiotherapy and medical therapies which consisted of standard antiplatelet, statin, anticoagulation, and antihypertensive drugs during the period spent in hospital.

2.4. MRI Acquisition. The resting-state fMRI data were acquired on a GE Signa HDxt 1.5 Tesla scanner (General Electric Medical System, Milwaukee, WI, USA) with an eight-channel head coil. To reduce head movements and scanner noises, the head of each patient was snugly fixed by a foam pad prior to the examination. After instructing the patients to keep awake, relaxed with eyes closed, and to remain motionless as much as possible, functional magnetic resonance imaging (fMRI) data were acquired by using an echo-planar imaging (EPI) sequence: TR/TE = 2, 000/40 ms, field of view = $240.0 \times 240.0\ mm^2$, flip angle = 90°, matrix = 64×64, voxel sizes = $3.75 \times 3.75 \times 5.0\ mm^3$, 32 axial slices, and no gaps. Each scan obtained 140 volumes continuously. A 3D high-resolution structural image acquisition was also conducted: 124 slices, TR/TE = 9.1/2.9 ms, field of view = $240.0 \times 240.0\ mm^2$, flip angle = 20°, matrix = 256×256, and voxel sizes = $0.94 \times 0.94 \times 1.2\ mm^3$.

2.5. Preprocessing of the fMRI Data. Image preprocessing was performed by using the SPM 12 (http://www.fil.ion.ucl.ac.uk/spm) software package. Prior to the preprocessing procedure, the first five volumes of the fMRI datasets of each patient were discarded to eliminate the magnetization equilibrium effects and allow the participants to adapt to the circumstances. Subsequently, spatial processing including time delay correction between slices, head motion realignment, spatial normalization to the standard brain space of the Montreal Neurological Institute (MNI) (resampled to a voxel size of $3.0 \times 3.0 \times 3.0$ mm), and spatial smoothing with 8.0 mm isotropic kernel was conducted.

2.6. Independent Component Analysis. Only the fMRI data of both rTMS groups was used to analyze the difference between HF-rTMS and LF-rTMS on the modulation of the motor network. With the preprocessed fMRI data, the GIFT software (http://icatb.sourceforge.net/) was used to conduct the group spatial independent component analysis (ICA) with the following stages: (1) two-stage data reduction of principal component analysis (PCA), (2) application of the ICA algorithm, and (3) back reconstruction using a dual-regression method to back reconstruct the individual independent components (ICs). To determine the number of ICs, dimension estimation on all patients of both rTMS groups was performed by using the minimum description length (MDL) criterion. Subsequently, the infomax algorithm was used in IC estimation. Then, following the reconstruction step, the individual specific IC maps were converted to a Z score. At last, the IC of the motor network was selected to be of interest for further analyses. Z maps of each group were then gathered for a random effects analysis using the one-sample t-test in SPM 12. Subsequently, to investigate the functional connectivity changes of the motor network after rTMS, the paired t-test analysis was used to compare the Z maps of the motor network of both groups between pre- and post-rTMS. Moreover, the same comparison of the Z maps between pre- and post-rTMS was conducted for each group, respectively, and also to understand the distinction of functional connectivity changes between the HF-rTMS and LF-rTMS groups.

2.7. Functional Connectivity Analysis of the Intramotor Network. Motor recovery of stroke has been demonstrated to be associated with the reorganization of the functional motor network [24]. Consistent dynamically increased regional centralities of the ipsilesional M1 within the motor network was also observed with the process of motor recovery [25]. Therefore, in this study, the core regions of the cortical motor network of bilateral hemispheres including M1, SMA, and premotor area (PMA) were mainly focused on in order to investigate the modulation of rTMS on the functional connectivities among these regions of the intramotor network. The peak coordinates of these core regions were identified and selected from the comparison results of the motor network obtained from ICA analysis between pre- and post-rTMS of both groups. Finally, a spherical region of interest (ROI) (radius = 5.0 mm) was defined and centered at each peak coordinate within the corresponding brain region.

Subsequently, the signal extraction, preprocessing, and functional connectivity analysis of the motor network were all completed in the Resting-State Hemodynamic Response Function Retrieval and Deconvolution (rsHRF) plugin (https://github.com/compneuro-da/rsHRF) in SPM [26]. By using this software package, the blood oxygenation level-dependent (BOLD) fMRI signal was deconvolved to minimize the variability of HRF [27]. The time series of all the voxels in each ROI was extracted from the preprocessed fMRI dataset and averaged as the representative time signal of the ROI. To minimize the effect of global drift, the time signal of each ROI was scaled by dividing each time point's value by the mean value of the whole brain image at that time point. After this, the scaled waveform of each signal was filtered by using a bandpass filter (0.01-0.08 Hz) to reduce the effect of low-frequency drift and high-frequency artifacts

related to head motion and physiological noise including respiration and cardiac cycle. The head motion parameters, white matter signals, and cerebrospinal fluid signals were then used as covariates of multiple linear regression. Subsequently, the Pearson correlation coefficients were calculated between the time signals of all ROIs and normalized to z -scores by using Fisher's r to z transformation. Statistically significant ($p < 0.05$) correlation coefficient was considered a valid connectivity and used to describe the edge of the motor network. For each patient, two motor networks were obtained pre- and post-rTMS. A paired t-test was employed to observe the significantly changed connectivities between regions after rTMS for the HF-rTMS group and LF-rTMS group separately.

2.8. Correlation Analysis. To further verify the consistent performance between the functional connectivity of the motor network and motor function, we computed the Pearson correlation coefficients between the values of functional connectivity changes and motor assessment score changes as well in each group. The statistical analysis was conducted by using a threshold of $p < 0.05$.

2.9. Statistical Analysis. Statistics for demographics and cognitive test scores were calculated with appropriate chi-squared (χ^2), ANCOVA, or Student's t-tests. Statistical parametric and nonparametric tests were used depending on the type of scale and nature of the variable distribution. ANCOVA with age and gender as covariates was performed to determine the main effect of rTMS, followed by post hoc two-sample t-tests for multiple comparisons. Paired t-tests were conducted to assess the changes of cognitive function postintervention within each group. The significance was set at $p < 0.05$.

3. Results

3.1. Behavioral Information. The demographic characteristics and neurological examinations of HF-rTMS, LF-rTMS, and sham groups are summarized in Table 1. The mean and standard deviation (SD) of age, the time since stroke (days), and the FMA, BI, and NIHSS of patients of pre- and post-rTMS are all provided in the table. There are no significant differences among the three groups in age, gender, time since stroke (days), or clinical performances at baseline. Compared to baseline, both the motor function and daily living ability postintervention were all significantly improved according to the results of the two-factor ANCOVA which revealed significant main effects of "time" for the FMA, BI, and NIHSS ($p < 0.001$). The significant interaction between "group" and "time" was also found for the FMA ($F = 13.023$, $p < 0.001$) and BI ($F = 6.021$, $p = 0.006$) scores. Post hoc t-tests revealed that NIHSS scores were significantly lower in both rTMS groups compared to the sham group (HF-rTMS vs. sham, $p = 0.028$; LF-rTMS vs. sham, $p = 0.020$). The paired t-test revealed significantly improved FMA, BI, and NIHSS scores in the three groups after rTMS treatment relative to pre-rTMS ($p < 0.05$). All the score changes of FMA, BI, and NIHSS scores after rTMS were bigger in the HF-rTMS group relative to LF-rTMS and sham groups. During the rTMS sessions, no discomfort was reported from any patients in three groups.

3.2. Changes of Functional Connectivity of the Motor Network. After the group ICA analysis, the spatial independent component image of the motor network was extracted for each patient. These image data of both HF-rTMS and LF-rTMS groups were used to investigate the influence of rTMS therapy on the functional connectivity of the motor network. Compared to pre-rTMS, the significantly increased functional connectivity was observed in bilateral M1, SMA, and contralesional PMA after rTMS ($p < 0.05$, AlphaSim correction, and cluster size > 197) (Figure 1 and Table 2). In addition, to further clarify the distinction of HF-rTMS and LF-rTMS on the modulation of functional connectivity of the motor network, respectively, the comparison between pre- and post-rTMS in the HF group and LF-rTMS group was performed separately. Significantly increased functional connectivity was observed in the ipsilesional M1, SMA, and PMA after HF-rTMS ($p < 0.05$, AlphaSim correction, and cluster size > 219) (Figure 2(a)). In contrast, the enhanced functional connectivities were observed in the contralesional M1 and bilateral SMA in the LF-rTMS group after rTMS ($p < 0.05$, AlphaSim correction, and cluster size > 213) (Figure 2(b)). Furthermore, decreased functional connectivity was detected in the bilateral SMA as well.

3.3. Changes of Functional Connectivities of the Intramotor Network. To validate the modulation of rTMS on the network pathway between brain regions of the motor network, the functional connectivity intramotor network was calculated with the selected peak coordinates in Table 2. The symmetric location homologous to the contralesional PMA (-33, -7, and 61) and SMA (9, 2, and 61) was selected for the two regions which did not show significant changes after rTMS. The comparisons of functional connectivity of the intramotor network pre- and post-rTMS within each group and between HF-rTMS and LF-rTMS groups after rTMS were also conducted. Figure 3 demonstrates statistically significant functional connectivity and changes of the motor network pre- and post-rTMS in the HF-rTMS group and LF-rTMS group and between two groups. The disconnectivity induced by stroke at baseline was basically recovered after rTMS, especially among the ipsilesional motor-related brain regions and between regions of the ipsilesional and contralesional hemisphere. Although most of the connectivity did not reach a statistically significant level, these findings revealed the reconnection within the motor network of the affected hemisphere and with the unaffected hemisphere after rTMS.

The significantly increased functional connectivities were detected between the ipsilesional M1, ipsilesional SMA, and contralesional PMA, between contralesional M1 and contralesional SMA, and between contralesional SMA, ipsilesional SMA, and contralesional PMA in the HF-rTMS group. No significant functional connectivity changes were observed in the LF-rTMS group. Significantly higher functional connectivity was found between ipsilesional M1 and contralesional PMA in HF-rTMS relative to the LF-rTMS

Table 1: Demographic, clinical, and motor test variables of stroke patients.

Variables		HF_group ($n = 11$)	LF_group ($n = 12$)	Sham_group ($n = 10$)	F/χ^2	p
Age		65.09 ± 5.84	63.58 ± 7.95	64.9 ± 6.23	0.168	0.846
Gender (F/M)		6/5	7/5	5/5	0.153	0.926
Time since stroke (days)		6.00 ± 2.37	5.42 ± 1.93	5.1 ± 1.79	0.528	0.595
FMA	Pre	38.45 ± 22.64	37.83 ± 15.06	36.70 ± 15.37	13.023	0.000
	Post	54.64 ± 19.82[a,b]	52.67 ± 19.98[a,b]	40.6 ± 16.33[a,b]		
BI	Pre	43.64 ± 25.31	45.42 ± 20.05	43.00 ± 15.49	6.021	0.006
	Post	61.82 ± 21.71[a,b]	59.58 ± 21.24[a,b]	47.50 ± 13.59[a,b]		
NIHSS	Pre	7.09 ± 2.77	5.75 ± 2.73	7.40 ± 1.96	2.852	0.073
	Post	3.27 ± 1.74[a]	3.17 ± 2.66[a]	5.40 ± 1.71[a]		

HF: high frequency; LF: low frequency; FMA: Fugl-Meyer Assessment; BI: Barthel Index; NIHSS: National Institutes of Health Stroke Scale; M: male; F: female. [a]The significant differences between pre- and post-rTMS with a paired t-test ($p < 0.05$). [b]The significant differences between groups from baseline to postintervention with repeated measures ANOVA ($p < 0.05$).

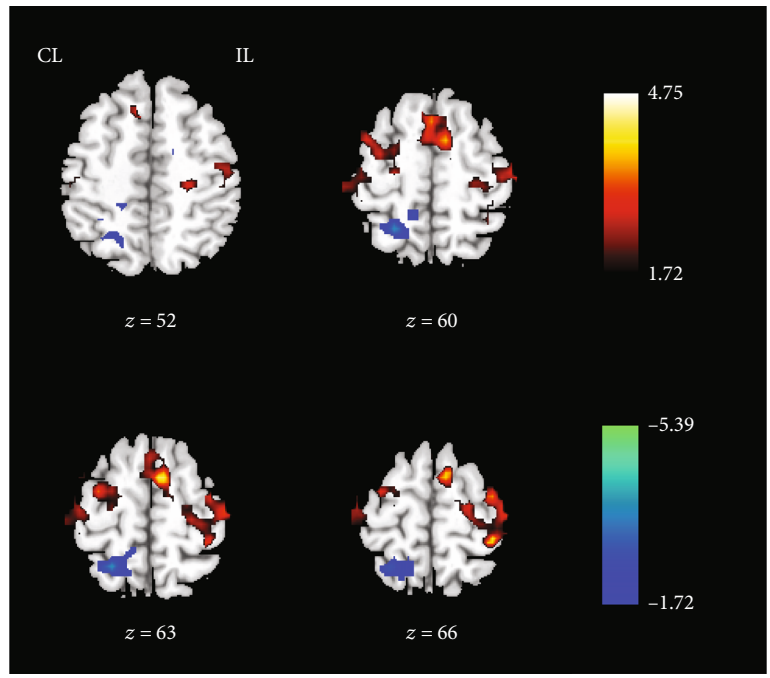


Figure 1: Functional connectivity changes of the motor network after rTMS treatment. CL: contralesional side; IL: ipsilesional side. The warm color indicates the increased functional connectivity, and the cold color indicates the decreased functional connectivity after rTMS.

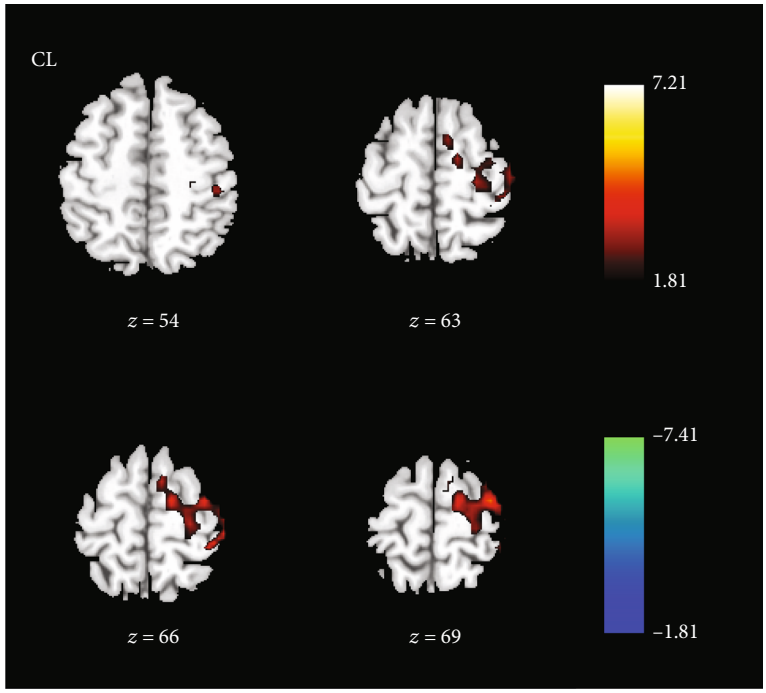
Table 2: Brain regions showing significantly changed functional connectivities in the motor network after rTMS in both rTMS groups.

Region	Side	T value	Cluster size (voxels)	MNI coordinate		
				x	y	z
M1	IL	4.11	298	-39	-37	64
M1	CL	2.55	123	45	-19	61
SMA	BL	4.27	187	-9	2	61
PMA	CL	3.34	151	33	-7	61

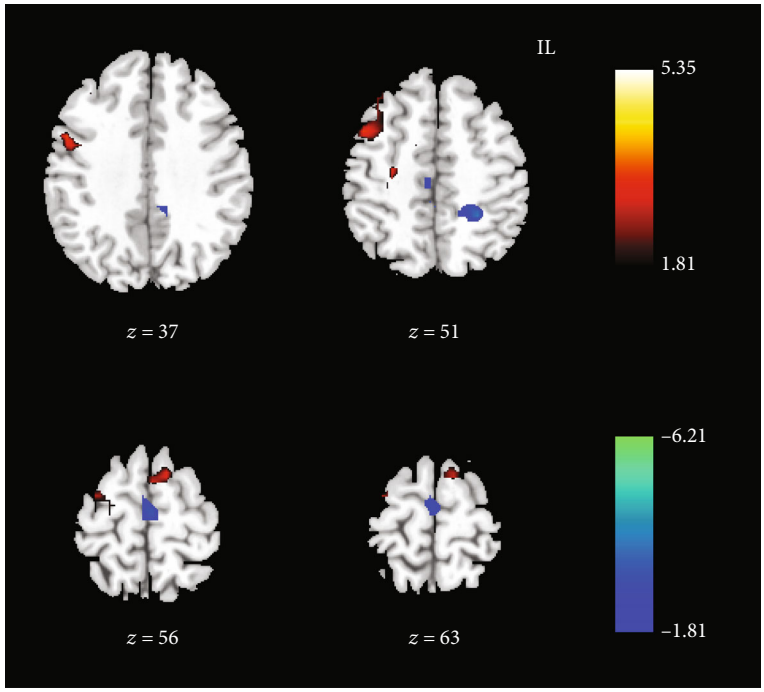
MNI: Montreal Neurological Institute; M1: primary motor cortex; SMA: supplementary motor cortex; PMA: premotor area; IL: ipsilesional side; CL: contralesional side; BL: bilateral side.

group as well. These findings suggest the modulation of rTMS on functional interactions among the motor brain regions within the affected hemisphere and interaction of bilateral hemispheres following treatment.

3.4. Relationship between Functional Connectivity and Motor Performance. To verify the relationship between the significantly changed functional connectivity and motor recovery alteration reflected by neurological examination, a Pearson correlation coefficient was calculated in both HF-rTMS and LF-rTMS groups. For the functional connectivity intramotor network, the increased functional connectivity between ipsilesional M1 and contralesional PMA ($r = -0.678$, $p = 0.022$) (Figure 4) was significantly negatively correlated with the



(a) HF group post vs. pre



(b) LF group post vs. pre

Figure 2: Functional connectivity changes of the motor network after HF-rTMS (a) and LF-rTMS (b) separately. CL: contralesional side; IL: ipsilesional side. The warm color indicates the higher functional connectivity, and the cold color indicates the lower functional connectivity in the HF-rTMS group.

NIHSS improvement in the HF-rTMS group. No significant correlation result was detected in the LF-rTMS group and other functional connectivities of the motor network. This result may indicate the reconnection between the brain regions which may contribute to the restoration of motor function after HF-rTMS.

4. Discussion

In this current study, both ICA and seed-based analyses were used to investigate the functional reorganization of the motor network of stroke patients with motor deficit after rTMS. The distinction between HF-rTMS and LF-rTMS on the

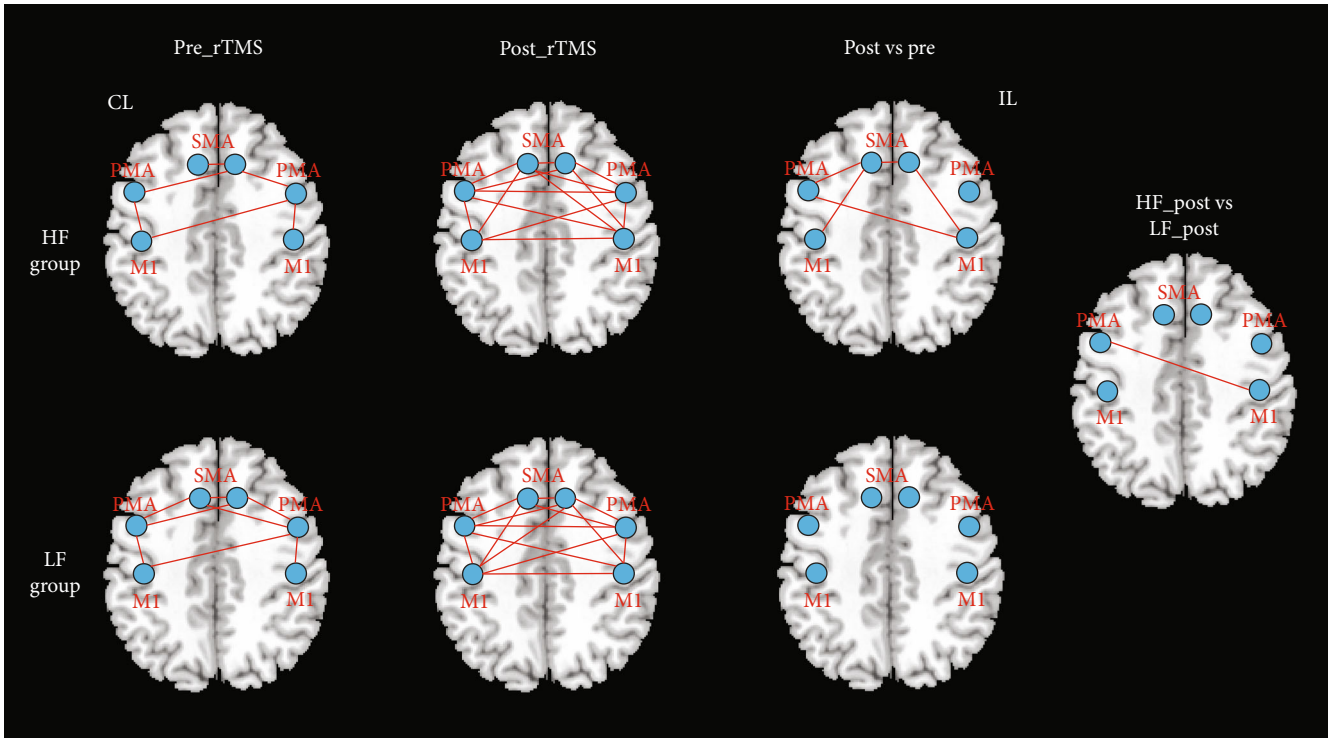


Figure 3: Significant functional connectivity intramotor network and changes after rTMS. HF: high frequency; LF: low frequency; CL: contralesional side; IL: ipsilesional side; M1: primary motor cortex; SMA: supplementary motor area; PMA: premotor area.

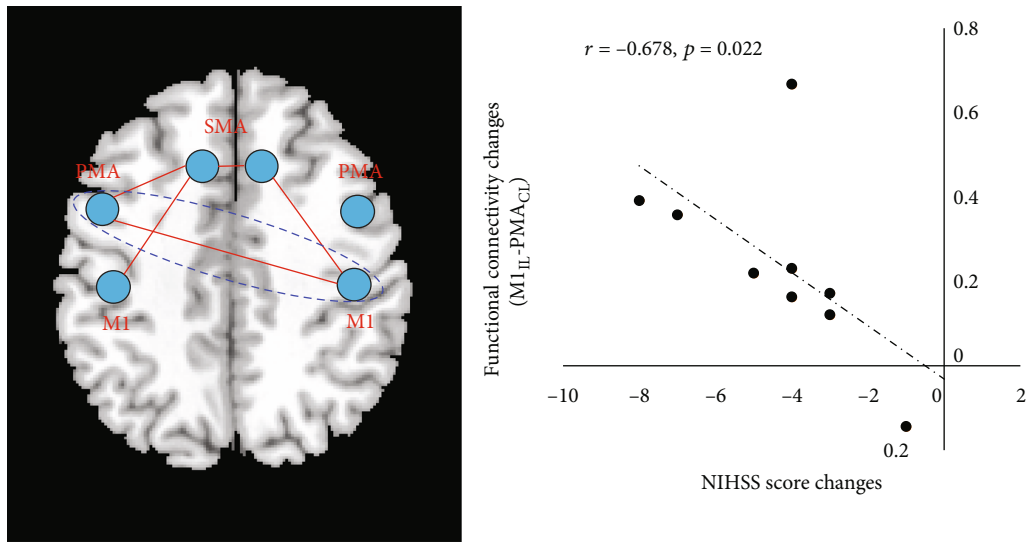


Figure 4: Pearson correlation between the changes of functional connectivity (between ipsilesional PMA and contralesional M1) and NIHSS score changes in the HF-rTMS group. M1: primary motor cortex; PMA: premotor area; SMA: supplementary motor area; IL: ipsilesional; CL: contralesional.

modulation of the motor network was further discussed. We found that HF-rTMS prominently increased the functional connectivity of the motor network in the ipsilesional hemisphere, whereas LF-rTMS mainly focused on the contralesional hemisphere. Moreover, the interaction between ipsilesional M1 and contralesional PMA and between bilateral SMA may contribute more during the motor recovery with HF-rTMS therapy. Our findings suggest that the distinct functional restoration and reorganization within the motor network of HF-rTMS and LF-rTMS both may underlie the motor recovery.

In our study, significantly improved motor function was detected in both HF-rTMS and LF-rTMS groups relative to baseline and sham groups. Furthermore, greater changes of

FMA, BI, and NIHSS were all found in the HF-rTMS group than in the patients in the LF-rTMS group. The positive effect of rTMS on the motor recovery and activities of daily living of stroke patients with motor dysfunction has been reported in several meta-analyses [7, 28, 29]. In accordance with our results, one of the meta-analyses also found that HF-rTMS is more effective than LF-rTMS, but not significant [28]. However, the opposite result was reported in another meta-analysis [7]. Therefore, future investigation with more studies is necessary to validate the result.

Consistent with the results of neurological examinations, significantly increased functional connectivity of the motor network was observed in both groups as well. Furthermore, the motor-related brain regions showing network changes were located in the ipsilesional hemisphere after HF-rTMS and in the contralesional hemisphere after LF-rTMS. These results could be explained with the distinct mechanisms of different modes of rTMS which suggested that HF-rTMS over the ipsilesional hemisphere could increase the cortical excitability of the damaged cortex; low-frequency rTMS over the contralesional hemisphere could potentially decrease abnormally increased inhibition to the lesioned M1 and promote the recovery of the damaged cortex [30]. Several comprehensive studies on motor recovery in early stroke patients showed that both HF-rTMS and LF-rTMS could increase motor-evoked fMRI activation of the ipsilesional motor area which were also positively significantly correlated with motor function at postintervention in M1 [31–33]. The increased fMRI activation in ipsilesional M1 was observed in patients with good motor outcome as well [31]. Therefore, both the excited rTMS over the ipsilesional M1 and the inhibitory rTMS over the contralesional hemisphere have shown promise in enhancing stroke patients' recovery [14].

Except for different motor network changes, more significant functional connectivities intramotor network was found in the HF-rTMS group between the ipsilesional motor cortex and contralesional motor areas. The increased functional connectivity between ipsilesional M1 and contralesional PMA was also observed significantly related to the motor improvement. Additionally, this connectivity was also found higher in the HF-rTMS group than in the LF-rTMS group. Several previous studies have proved the crucial role of contralesional PMA, in particular, the dorsal PMA, in motor function and motor recovery. After stroke, fMRI investigations showed more activation in the contralesional PMA during the movement of the affected limb and were prominent in patients with poor motor recovery [34–36]. Such activity changes may imply the associated motor recovery. Inhibitory low-frequency rTMS over contralesional PMA also could slow the affected finger movement, in particular in more impaired patients, suggesting the functional recruitment of contralesional PMA in motor recovery [36]. This results also demonstrated its adaptive compensation for an injured motor cortex after stroke. Further studies on behavior, neuroimaging, and neuropsychological validate that motor impairment and recovery after stroke could be explained with the specificity of PMA to the process of action selection [37–39]. Moreover, a concurrent TMS-fMRI study further found the physiological influence of contralesional PMA on ipsilesional M1 [40]. Furthermore, stronger promotional influence between them was associated with greater clinical and neuropsychological impairment during hand grip in stroke patients. Dual-site TMS studies also found that TMS-induced activation changes in contralesional PMA have a causal impact on ipsilesional M1 at short latencies [41, 42], so a likely alternative route by which contralesional PMA could exert control over ipsilesional finger movement is via interhemispheric connections with contralateral M1 [43]. Therefore, these evidences suggest that contralesional PMA may be positioned to mediate functional recovery of motor function after stroke. The finding of significantly increased functional connectivity between ipsilesional M1 and contralesional PMA after rTMS may be explained by these above-mentioned theories and prove its contribution to motor recovery during high-frequency rTMS therapy.

Significant functional connectivity between ipsilesional and contralesional M1 was also observed after rTMS in both HF-rTMS and LF-rTMS groups, which was impaired after stroke. A previous study reported that increased functional connectivity between bilateral M1 was significantly correlated with the improvement in the upper limb section of FMA which was detected after the motor imagery training combined with conventional rehabilitation therapy [44]. Another study with acupuncture treatment also observed increased functional connectivity between bilateral M1 [45]. In addition, prior to treatment, several studies found significantly decreased interhemispheric functional connectivity between ipsilesional M1 and contralesional M1 after stroke [4, 45–47]. One study suggested that the transcallosal connections between bilateral M1 was also associated with motor recovery [48]. Therefore, our finding may indicate the efficacy and modulatory effect of high- and low-frequency rTMS on the motor network.

In considering the whole brain, stroke induces interhemispheric changes and not just the neural activity and functional connectivity in the affected and unaffected hemisphere [49]. Therefore, according to the model of interhemispheric interaction, motor recovery after stroke may be linked to rebalancing of asymmetric interhemispheric excitability and connectivity. This theory also confirmed the rationale of neuromodulation techniques to suppress unaffected motor cortex excitability and facilitate affected motor cortex excitability [50]. Noninvasive treatments including rTMS and transcranial direct current stimulation (tDCS) were both mainly performed to restore abnormal interhemispheric balance by facilitating ipsilesional M1 excitability or by inhibiting contralesional M1 excitability [17, 22, 51, 52]. They observed slightly but not significantly increased intrahemispheric connectivity of the ipsilesional M1 after stimulation with both rTMS and tDCS [17, 53]. This is in accordance with our results between the ipsilesional M1 and PMA. The functional role of SMA for motor recovery has been proven for a long time. The functional connectivity increase between the ipsilesional M1 and contralesional SMA demonstrated the efficacy of rTMS. Moreover, significant changes in neurochemicals were detected in the affected M1 as well when stimulating the unaffected M1. They believed that interhemispheric connectivity is also particularly important in

functional recovery after stroke. In our study, more interhemispheric functional connectivity changes were observed which may indicate that functional compensation from the contralesional hemisphere may play a more important role during motor recovery. rTMS may realize its effect by modulating the functional connectivities between ipsilesional and contralesional motor-related brain areas. Direct intervention of HF-rTMS over the affected M1 may contribute more to the motor recovery which could explain the more increased functional connectivity of the motor network.

Some limitations exist in our study. First, a relatively small sample size was used in our study which may influence the results. We only included 11 subjects for the HF-rTMS group, 12 subjects for the LF-rTMS group, and 10 subjects for the sham group. It is difficult to ensure the cohorts of patients, but, in this study, there was no significant difference among the three groups in demographic characteristics, neurological examinations, and functional connectivity at baseline. Studies with more stroke patients are needed to verify our results. Second, only the core regions of the motor network were selected to characterize the functional reorganization. Subcortical brain regions also could be considered to fully understand the network changes after rTMS. Third, after completing the arranged sessions, the durability and influence on the motor network of HF-rTMS and LF-rTMS interventions were not made with the postintervention measurements.

Therefore, further studies with large sample sizes and long-term follow-up assessments are needed to interpret and verify the results more accurately.

5. Conclusions

Our study demonstrates that both HF-rTMS and LF-rTMS interventions could promote the motor rehabilitation in patients with stroke. Strikingly, HF-rTMS over the ipsilesional M1 may be more beneficial to the reorganization of the motor network and remodeling of motor cortical plasticity which realize greater contribution to the motor recovery.

Data Availability

The behavioral data used to support the findings of this study and the statistical analysis results are included within the supplementary information file. The data of fMRI used to support the findings of this study have not been made available because of the large number of original image files.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Zhiwei Guo and Yu Jin contributed equally to this work.

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Supplementary Materials

Original data of the basic information and behavioral scores of enrolled patients. *(Supplementary Materials)*

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Research Article

Functional Correlates of Action Observation of Gait in Patients with Parkinson's Disease

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Background. Action observation (AO) relies on the mirror neuron system (MNS) and has been proposed as a rehabilitation tool in Parkinson's disease (PD), in particular for gait disorder such as freezing of gait (FOG). In this study, we aimed to explore the brain functional correlates of the observation of human gait in PD patients with (FOG+) and without (FOG-) FOG and to investigate a possible relationship between AO-induced brain activation and gait performance. *Methods*. Fifty-four participants were enrolled in the study (15 PD FOG+; 18 PD FOG-; 21 healthy subjects (HS)) which consisted of two tasks in two separate days: (i) gait assessment and (ii) task-fMRI during AO of gait. Differences between patients with PD (FOG+ and FOG-) and HS were assessed at the level of behavioral and functional analysis. *Results*. Gait parameters, including gait velocity, stride length, and their coefficients of variability (CV), were different in PD patients compared to HS, whereas gait performance was similar between FOG+ and FOG-. The PD group, compared to HS, presented reduced functional activation in the frontal, cingulum, and parietooccipital regions. Reduced activity was more pronounced in the FOG+ group, compared to both HS and FOG- groups. Gait variability positively correlated with precuneus neural activity in the FOG+ group. *Discussion*. Patients with PD present a reduced functional activity during AO of gait, especially if FOG+. A baseline knowledge of the neural correlates of AO of gait in the clinical routine "on" status would help for the design of future AO rehabilitative interventions.

1. Introduction

The observation of someone performing an action recruits brain area that is activated also during the action execution. The physiological bases of this phenomenon rely on the mirror neuron system (MNS) [1, 2]. The MNS has been identified as the neural substrate for action observation (AO) training, observation plus repetition of actions, which has been proposed as a rehabilitation strategy in neurological disorders, including Parkinson's disease (PD) [3]. However, whether the MNS, and thus the efficacy of AO, is preserved or altered in patients with PD is still controversial [4]. Indeed, voluntary movement imitation seems to be preserved [5] and movement observation is accompanied by bilateral beta reduction in subthalamic power and cortico-subthalamic coherence [6]. On the contrary, the modulation of motor-evoked potentials [7] as well as the event-related mu rhythm desynchronization [8] during AO has been showed to be impaired in PD.

Gait disorders are frequent in PD patients, and among them, freezing of gait (FOG) affects up to 54% of patients [8]. However, in spite of their major effects on disability, their neural correlates remain quite unknown. Most of the studies exploring the neural correlates of FOG in PD have been performed with fMRI by a motor imagery task, revealing the involvement of the mesencephalic locomotor regions, basal ganglia, and supplementary motor and parietal cortices [9, 10]. The activation in the premotor, parietal, and

pontomesencephalic regions in patients with FOG (FOG+) was modulated by the antiparkinsonian treatment [11].

It has been demonstrated that AO training is effective in improving gait disturbances such as FOG [12]. Agosta and coworkers showed that PD patients with FOG exhibited a clinical improvement and increased recruitment of cortical areas involved in motor and attentional control, after a training based on AO [13]. However, in this study, only PD patients with FOG were included, while data in the literature about the neural correlates of AO of gait comparing PD patients with and without FOG are still missing. In addition, it should be considered that most previous studies have explored brain activity during gait in PD patients in the "off" state [14–16]. Although this may unveil the functional reorganization and reflect more accurately the pathophysiological substrate of the disease, it prevents the comprehension of the neural functional mechanisms underlying behavioral performance in the everyday life of PD patients. Thus, a better understanding of the functional changes occurring during AO of gait in patients with or without FOG under dopaminergic treatment would help lay the grounds for the design of more effective rehabilitative strategies for PD. This prompted us to design a functional MRI (fMRI) study (i) to explore the neural structures recruited during the observation of human gait in PD patients and (ii) to detect possible differences between PD patients with (FOG+) and without (FOG-) FOG while in the "on" state. Further, we aim also (iii) to assess whether there is a relationship between AO-induced brain activation and gait performance.

2. Methods

2.1. Subjects. A total of 54 subjects, 33 PD subjects and 21 healthy subjects (HS), were prospectively enrolled in the study. Participants with PD were recruited at the Department of Neuroscience, University of Genova. Healthy subjects were recruited from a local community as the control group. The study was approved by the regional ethical committee, and written informed consent was obtained from each participant prior to study entry. Common inclusion criteria were as follows: (i) >25/30 at the Mini-Mental State Examination (MMSE) and (ii) able to walk for 5 minutes unassisted. PD patients were included if they had idiopathic PD, as defined by the UK Brain Bank criteria, were in Hoehn and Yahr stage II–III, and were medically stable for at least 3 months prior to the study. Exclusion criteria, based on patients' report and medical records, for all participants included the following: (i) history of neurologic disorders other than PD, (ii) psychiatric comorbidity (e.g., major depressive disorder as determined by DSM IV criteria), (iii) contraindications to magnetic resonance imaging (MRI) exam, and (iv) visual impairments that could hinder task functional MRI (task-fMRI) acquisition.

2.2. Study Design and Procedures. The study consisted of two separate experimental sessions (gait evaluation and AO task-fMRI) performed by each participant in two different days. On the first day, demographic and clinical characteristics were collected, and then, participants were randomly assigned to either gait assessment evaluation first or AO task-fMRI first by a computerized block randomization, with a block size of 6. On the second day (≈after 7 days), subjects performed the other part of the study protocol. All PD patients were under treatment with dopaminergic therapy, and evaluations took place during the "on" state (≈1 hour after taking antiparkinsonian medications).

2.3. Demographics and Clinical Evaluations. Age, sex, and education were recorded for each participant along with other subject characteristics. For PD participants, disease severity was evaluated with section III of the MDS-Unified Parkinson Disease Rating Scale [17]. The Montreal Cognitive Assessment (MoCA) was used to evaluate global cognitive dysfunction [18]. The new FOG questionnaire [19] and the rapid 360-degree turn in place task [20] were used for evaluating the presence and the severity of FOG.

2.4. Action Observation: Task-fMRI. The MRI protocol was aimed at assessing the functional activity during AO of gait; we chose a block fMRI design with 30 seconds of rest (one block) followed by 30 seconds of task, 8 blocks total. Subjects were required to watch a third-person video clip representing human walking, inside the magnetic resonance scanner. Participants watched the video clip by looking at the mirror positioned on the head coil. The mirror reflected the human gait video displayed on a screen placed inside the magnet room, located ≈1 m far from the bottom of the scan. A custom-made Matlab® software synchronized video clip onset with fMRI acquisition.

2.5. Functional MRI Image Acquisition and Preprocessing. Images were acquired on Signa Excite 1.5 MRI (Signa Excite General Electric Healthcare, USA) with an 8-channel phased-array head coil. The MRI protocol included a T2-weighted sequence (TR/TE = 2340/102 ms, voxel size: 0.94 $\times$ 0.94 $\times$ 4 mm^3), Fast Spoiled Gradient Echo (FSPGR) 3D T1-weighted sequence (TR/TE = 11.70/5.12, voxel size: 1 $\times$ 1 $\times$ 1 mm^3), and a single-shot echo-planar imaging (EPI) sequence (TR/TE = 3000/60 ms, slice thickness = 4 mm, pixel size = 3.75 mm^2) for task-fMRI during action observation of gait.

The initial preprocessing step included the despiking (detection and reduction of extreme time series outliers by fitting a smooth curve insensitive to extreme outliers to the data), performed in AFNI (https://afni.nimh.nih.gov) [21]. Brain extraction was performed with FreeSurfer skull stripping on the T1-weighted anatomical sequence. The other preprocessing steps were performed using FSL [22] (FMRIB's Software Library, https://fsl.fmrib.ox.ac.uk/fsl/fslwiki) as implemented in FEAT [23], including removal of the first 3 volumes, motion correction using MCFLIRT (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MCFLIRT) [24], slice timing correction for regular ascending acquisition (using Fourier-space time series phase shifting), spatial smoothing (Gaussian kernel, full width at half maximum of 6 mm), grand-mean intensity normalization of all volumes by a single multiplicative factor, and high-pass temporal filtering (Gaussian-

weighted least-squares straight-line fitting, sigma = 30 sec). T1-weighted brain images were segmented into white matter (WM), grey matter (GM), and cerebrospinal fluid (CSF) using FAST; then, the WM and CSF masks were registered to the functional space and the average of the raw time series within each mask was derived in order to obtain the nuisance signal from WM and CSF. Boundary-based registration BBR [25] was used to register each individual functional data to the corresponding T1-weighted brain image. Then, linear affine 12-degree of freedom registration was performed to register each subject's T1-weighted brain to the standard space (MNI152 brain template, voxel size: 2 mm^3 [21].

2.6. Gait Evaluation. Participants were required to walk at their comfortable speed (labeled as normal walking (NW)) on a sensorized mat (GAITRite®) for 1 minute. To ensure that steady speed walking was recorded, 2 meters were added at the beginning and at the end of the GAITRite during gait task. Spatiotemporal parameters were analyzed with the ProtoKinetics Movement Analysis Software. Gait assessment protocol is depicted in Figure 1.

The ProtoKinetics software was used for analyzing the spatiotemporal gait parameters. Cadence (number steps × minutes), gait velocity (GV), stride length (SL), and their coefficients of variability (CV) were then determined. Gait parameters were obtained from all steps recorded during the task.

2.7. Statistical Analysis

2.7.1. Demographics, Clinical Data, and Gait Assessment. Analyses were performed using Statistical Package for the Social Sciences (SPSS) version 22, and means and standard deviations (SD) were calculated for all dependent variables. Gender differences among groups (PD FOG+, PD FOG-, and HS) were assessed using the chi-square procedure. For age and education, group differences were assessed by the nonparametric Kruskal-Wallis test and group-to-group comparison was performed using the Mann-Whitney U test. For gait kinematic parameters that were normally distributed, a one-way ANOVA was used to perform group comparison. For clinical data, the comparison between the PD FOG+ and PD FOG- groups was done using an unpaired t-test. p values < 0.05 were considered significant.

2.7.2. Functional MRI. One explanatory variable (EV) was defined to model the on-off periods of the task (action observation (AO)) and convolved with the hemodynamic response function (HRF), to detect task-related activity. The 24 motion parameters calculated during motion correction were added as confound EVs together with the mean CSF and WM signals.

A one-sample t-test was used to model group mean activation for both PD patients and HS. Differences between the two groups were investigated using a two-sample unpaired t-test, adding age and gender as covariates. Moreover, to test for significant differences among the FOG+, FOG-, and HS groups, ANCOVA, with age and gender as covariates, was used.

Results were converted to Z values and then thresholded at $Z = 2.3$ for cluster formation and significance threshold corrected for multiple comparisons ($p < 0.05$).

The correlations between brain activations of HS or PD patients (both FOG- and FOG+ groups) and the behavioral measures, in particular GV, SL, and their CV (i.e., GV-CV and SL-CV), were modeled separately with age and gender as covariates. Z-maps were thresholded at $Z \geq 2.3$ for cluster formation, followed with a significance threshold of $p = 0.05$ (cluster corrected using the Gaussian Random Field Theory). Brain functional activations were labeled using the Eickhoff atlas (SPM Anatomy toolbox) [26].

3. Results

3.1. Demographics and Clinical Data. Two PD subjects could not complete the MRI examination. Moreover, two patients were excluded from the analysis due to gait data corruption. Thus, results were obtained from 50 participants (29 PD and 21 HS). Twelve PD subjects were confirmed to experience FOG, by the new FOG questionnaire [19] and the rapid 360-degree turn in place task [20], whereas the rest of the participants ($n = 17$) were classified as FOG-. At the end of the recruitment phase, age, sex, and education levels were similar among the three groups (PD FOG+, PD FOG-, and HS; p always > 0.05). As expected, a significant difference was found for MoCA score among the three groups ($p < 0.001$). Post hoc analysis revealed that the score was significantly lower in both groups of PD participants compared to the HS group (p always < 0.001), but similar between PD patients with and without FOG ($p = 0.131$).

For clinical data, statistical analysis did not show a significant difference for disease duration ($p = 0.633$), H&Y stage ($p = 0.061$), MDS-UPDRS motor part ($p = 0.090$), and Levodopa Equivalent Daily Dose (LEDD, $p = 0.087$) between the PD FOG+ and PD FOG- groups. All the details for demographic, clinical characteristics, and statistics are reported in Table 1.

3.2. Action Observation (AO) Task-fMRI

3.2.1. Single Group Activations. During the AO task, the whole PD group showed several clusters of activation at the level of the occipital and temporal regions, inferior and superior parietal lobule (IPL and SPL, respectively), and precentral gyrus, in both hemispheres. HS activated at the level of the temporal and occipital regions bilaterally, bilateral SPL and intraparietal sulcus (IPS), left pre- and postcentral gyrus, and superior frontal gyrus (Figure 2(a); Table S1 Supplementary Information). When the FOG- and FOG+ groups were investigated separately, the former showed activity at the level of the right temporal and frontal regions and bilateral occipito-parietal areas, while the latter activated only at the level of the occipital regions (Figure 2(b); Table S2 Supplementary Information).

3.3. Subgroup Comparison

3.3.1. PD vs. HS. When the PD and HS groups were compared, HS showed a significantly greater activation at the

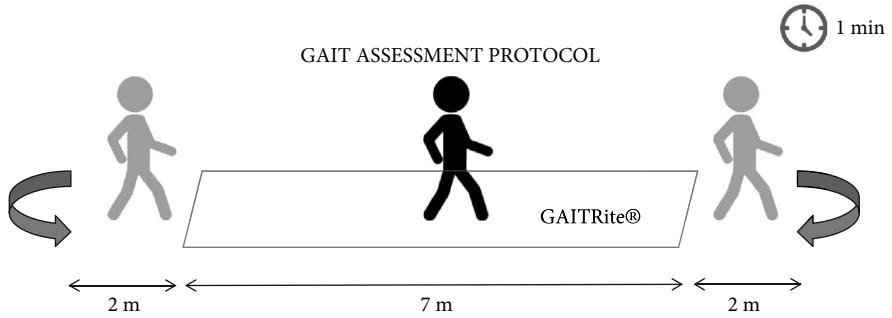

Figure 1: Gait assessment protocol. PD participants walk at their comfortable speed on GAITRite® for 1 minute.

Table 1: Demographics, clinical characteristics, and gait parameters of participants.

	HS ($n = 21$)	PD FOG- ($n = 17$)	PD FOG+ ($n = 12$)	p value
Demographic and clinical characteristics				
Age (y, mean ± SD)	64.62 ± 13.52	68.67 ± 4.60	72.00 ± 4.51	$p = 0.08$
Education (y, mean ± SD)	11.16 ± 6.37	9.62 ± 6.44	10.03 ± 4.26	$p = 0.19$
MoCA (score, mean ± SD)	28.81 ± 1.01	26.06 ± 2.79	24.33 ± 2.71	$p < 0.01$
Disease duration (y, mean ± SD)	—	9.24 ± 3.91	10.93 ± 3.54	$p = 0.633$
H&Y (stage, mean ± SD)	—	1.86 ± 0.46	2.20 ± 0.62	$p = 0.061$
MDS-UPDRS III (motor score, mean ± SD)	—	19.11 ± 9.37	26.75 ± 14.09	$p = 0.090$
LEDD	—	558.33 ± 252.99	409.911 ± 197.87	$p = 0.105$
Gait parameters				
Gait velocity (cm/s, mean ± SD)	129.95 ± 15.70	113.83 ± 21.41	113.95 ± 20.22	$p = 0.018$
Gait velocity CV (mean ± SD)	3.46 ± 0.91	4.49 ± 1.86	4.77 ± 1.53	$p = 0.024$
Stride length (cm, mean ± SD)	133.96 ± 13.94	122.17 ± 15.32	120.27 ± 13.01	$p = 0.012$
Stride length CV (mean ± SD)	2.20 ± 0.61	3.13 ± 1.06	3.32 ± 1.37	$p = 0.003$
Cadence (n steps × min, mean ± SD)	113.94 ± 9.31	110.00 ± 10.89	112.36 ± 15.08	$p = 0.575$

HS: healthy subjects; PD: Parkinson's disease; FOG-: patients without freezing of gait; FOG+: patients with FOG; n: number; y: years; SD: standard deviation; MoCA: Montreal Cognitive Assessment; H&Y: Hoehn and Yahr; MDS-UPDRS: Movement Disorder Society-Unified Parkinson Disease Rating Scale; LEDD: Levodopa Equivalent Daily Dose; CV: coefficient of variability.

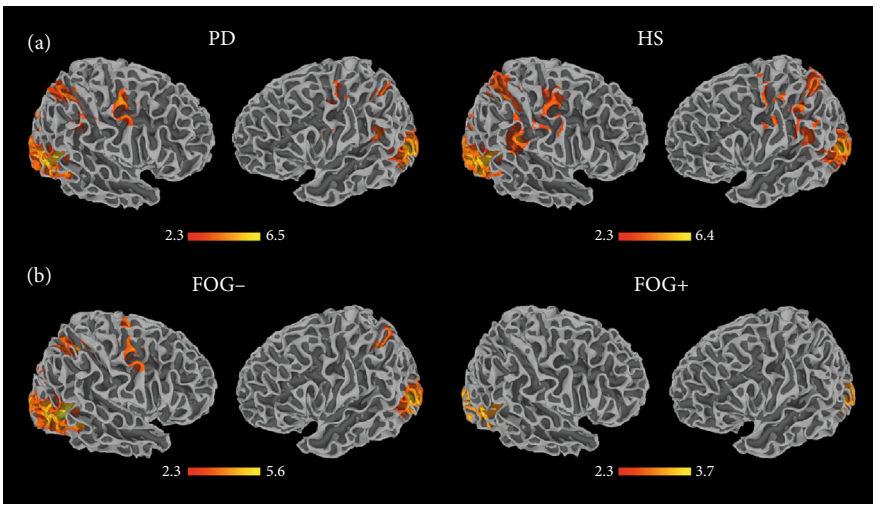

Figure 2: Cortical activity during AO of gait in the (a) whole group of PD patients and in the HS group and in (b) FOG- and FOG+ patients. The results are cluster corrected for multiple comparisons ($Z \geq 2.3$, $p < 0.05$) and are shown in MNI space.

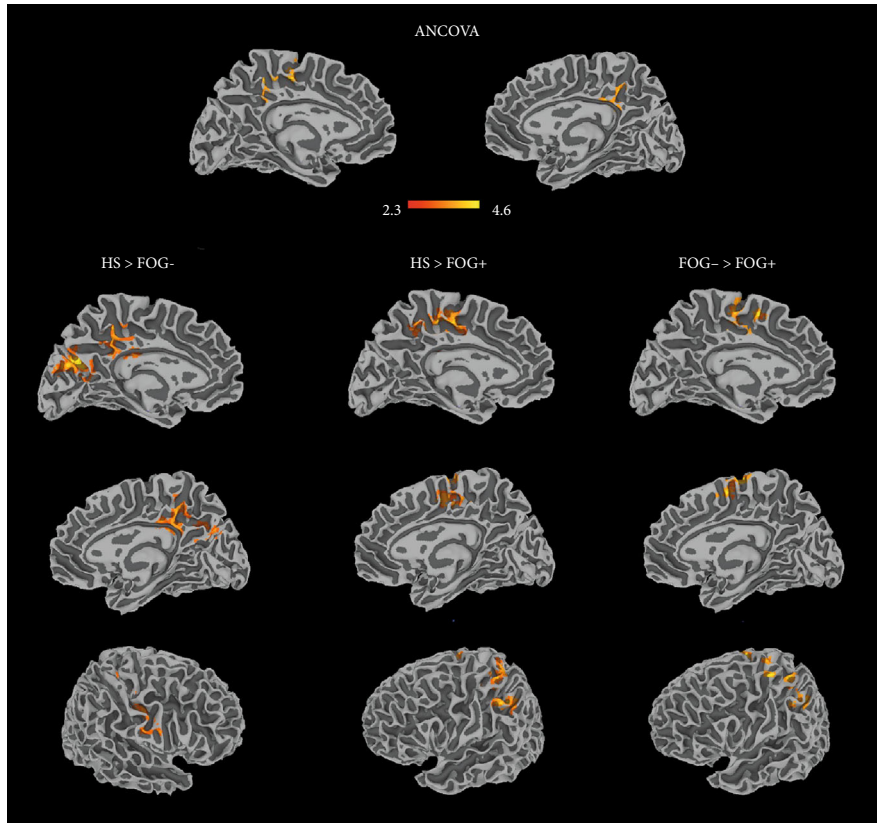

Figure 3: Results of the ANOVA analysis (top) and comparison between subgroups. The results are cluster corrected for multiple comparisons ($Z \geq 2.3$, $p < 0.05$) and are shown in MNI space.

level of the cingulate cortex, posterior medial frontal cortex (PMFC), occipital regions, and the precuneus (Table S1 Supplementary Information).

3.3.2. PD FOG+ vs. PD FOG- vs. HS (ANCOVA). The analysis revealed a significant difference among the three groups at the level of the left posterior-medial frontal cortex and cingulate cortex. Both FOG- and HS showed an increased activity in the bilateral PMFC, in the left IPL, and in the postcentral gyrus compared to FOG+ participants. Moreover, HS showed two clusters of greater activation, compared to FOG-, at the level of the left occipital regions, right precuneus, left cingulate cortex, and right pre- and postcentral gyrus (Figure 3; Table S3 Supplementary Information).

3.4. Gait Performance. As expected, statistical analysis revealed significant differences between the two groups (PD vs. HS) for most of the kinematic parameters obtained during gait task. One-way ANOVA revealed that GV and SL were different among groups ($p = 0.018$ and $p = 0.012$, respectively). Post hoc analysis showed that both FOG+ and FOG- participants had a reduced gait speed ($p = 0.024$ and $p = 0.012$, respectively) and shorter steps ($p = 0.011$ and $p = 0.014$, respectively) compared to HS. Regarding variability, GV-CV and SL-CV were significantly higher (i.e., worse) in patients with PD compared to HS participants (GV-CV $p = 0.024$; SL-CV $p = 0.003$). Post hoc analysis revealed a higher variability in FOG+ and FOG- patients compared to HS (GV-CV: FOG+ vs. HS ($p = 0.015$) and FOG- vs. HS ($p = 0.032$); SL-CV: FOG+ vs. HS ($p = 0.003$) and FOG- vs. HS ($p = 0.006$)). In the FOG+ group, no significant correlation was found between gait parameters and FOG-Q score.

3.5. Neuroimaging-Behavioral Correlations. When brain activations were correlated with kinematic parameters obtained during normal walking task, significant correlations were found in the FOG+ group. Specifically, increased activity at the level of the precuneus cortex was associated with higher SL-CV and GV-CV values (Figure 4, Table S4 Supplementary Information). Statistical analysis did not reveal any significant correlation between cluster significant activations and FOG-Q score or total MDS-UPDRS. Finally, no significant relationships between brain activations and gait parameters were found for the FOG- or HS group.

4. Discussion

In this study, we investigated the neural mechanisms underlying AO of gait and the possible association between brain activity and walking performance in PD patients with and without FOG in the on state, under dopaminergic treatment. Three main findings were observed. First, patients with PD present reduced brain activation during AO of normal walking. Second, functional reorganization occurs both in FOG- and FOG+ patients, being more evident in the latter group.

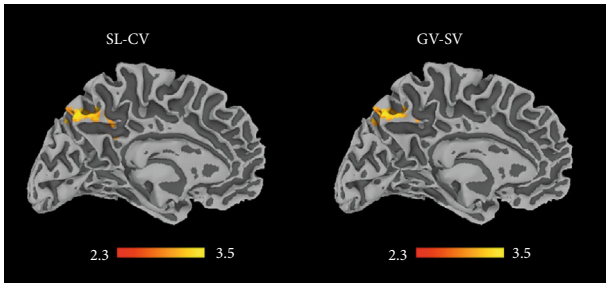

Figure 4: Neuroimaging-behavioral correlations, in particular correlations of brain activity during AO of gait and (a) SL-CV or (b) GV-CV. The results are cluster corrected for multiple comparisons ($Z \geq 2.3$, $p < 0.05$) and are shown in MNI space.

Third, in PD FOG+ participants, activity in the precuneus was associated with spatiotemporal parameters of gait.

Although AO is emerging as a tool for rehabilitation of PD symptoms, including FOG, the neural correlates of AO of gait in FOG+ vs. FOG- patients under dopaminergic treatment remain largely unexplored. We showed, in both HS and PD groups, a cortical activation at the level of the occipital, parietal, and frontal areas during the observation of walking. In particular, the pattern of activity we observed in HS is similar to the one reported by Iseki et al. [27]. However, cortical activation was significantly reduced in PD patients, when compared to HS at the level of the PMFC, of occipital areas, and of the precuneus.

In HS, both the occipital areas and the precuneus have been found to be active during observation of gait with precuneus being involved in the spatial control of motor behavior [28]. Both these areas were hypoactivated during real gait in patients with PD, compared to HS [29]. Besides, PMFC is one of the brain areas most consistently associated with gait [27, 29] and found to be affected in patients with PD [28]. Thus, our results confirm the impaired functionality of frontoparietal areas related to gait in patients with PD, even during AO of gait. Finally, previous studies have already shown decreased activity of temporo-occipital regions in PD patients [30, 31] and also in PD with FOG [32] suggesting a possible deficit of visuospatial skills in PD.

When FOG+ and FOG- patients were considered separately, we found that the FOG+ group showed brain activation only at the level of occipital cortex and, compared to both FOG- and HS, presented a reduced activity at the level of association regions such as PMFC and IPL, while the main differences between HS and FOG- was at the level of occipital and primary sensorimotor cortices. Previous studies found a reduced activity in FOG+ patients, compared to FOG- [10], in particular at the level of the supplementary motor area and parietal regions [9] during motor imagery of gait. These results are supported by a recent resting-state functional connectivity study suggesting that FOG might reflect a "widespread increase in intrinsic connectivity within networks in the frontal and parietal areas and basal ganglia as well as a functional disruption between networks implicated in executive and dorsal attention functions" [33]. With this study, we confirm that FOG- and FOG+ differ also in the neural correlates underlying AO of gait and in particular that FOG+ patients present with a more impaired activation.

A previous study on AO in PD patients with FOG [13] revealed a reduced activity in the precentral and SMA areas, in comparison with HS. The preserved activation of PMFC during AO of gait, which we found in FOG- but not in FOG+ patients, adds a missing piece, confirming the involvement of this area in the functional reorganization subtending FOG [34]. Furthermore, the IPL has been described to play a role in the representation of actions triggered by sensory stimuli, including the visual inputs [3, 35]. Therefore, a dysfunction in this area may result in altered sensory input integration and a misrepresentation of the action contributing to the occurrence of FOG. This could be also one of the mechanisms underlying the effectiveness of AO training in improving FOG [12].

Related to the impact of dopaminergic medication on action observation network, a couple of studies analyzed changes in local field potentials recorded from the subthalamic nucleus (STN) during movement observation in on and off conditions [6, 36]. Movement observation was associated with significant changes in the beta oscillatory activity in the STN of PD patients. Particularly, there was a movement observation-related decrease in beta activity in the STN. This decrease, although smaller than those observed during the movement execution, had similar characteristics: it had the same relative amplitude in "on" and "off" and was bilateral and coherent with cortical activity [6]. Differently, the movement-related gamma increase was observed only in the movement execution condition and was modulated by dopaminergic therapy uptake [6]. Overall, these studies confirm what has been suggested related to dopaminergic modulation of network dynamics: dopaminergic medication may induce improvement of basic motor performance by selectively modulating the connectivity in premotor loops at the cortical level as well as cortico-subcortical interactions, but it is not able to efficiently compensate for higher motor control requiring executive functions [37]. In this view, during action observation, different studies suggested the involvement of a complex cortical-subcortical network in order to understand the context, the congruency, and the features of the motor act [38], spreading over association areas that are involved in executive functions. Therefore, the differences observed in the current study could suggest that antiparkinsonian treatment is not sufficient to normalize the neural activity underlying AO in patients with PD, in particular if FOG+ where the impairment of cognitive association areas is more prominent [39]. Lastly, based on the results of Agosta and colleagues [13], it seems the reduced activation of the PMFC in FOG+ compared to controls is not influenced by the antiparkinsonian treatment. Nonetheless, further investigations comparing functional activity in patients in the "on" vs. "off" state during AO of gait are needed to validate these results.

Overall, our results confirm an impaired cortical activation in PD patients, compared to HS, even under the effects of antiparkinsonian medication. Both PD patients with and without FOG undergo a change in the functional connectivity subtending the AO of gait, but a wider modification

occurs in PD patients presenting FOG. The knowledge of the pattern of brain activity during AO of gait under the effect of medications could help in understanding the functional modulation shaped by AO training and therefore in designing the most appropriate rehabilitation protocols.

It is worth noting that in our study we did not find significant differences in the spatiotemporal parameters of gait between the two PD groups, even if it is common knowledge that gait features may differ between PD with and without FOG [37, 38]. To elicit FOG episode during a clinical evaluation is not easy. Indeed, in many patients suffering from FOG, despite experiencing FOG during daily living, it is often difficult to observe FOG episodes during examinations conducted in clinical settings. The presence of FOG needs to be often provoked by FOG-provoking tasks or to be measured during more complex circumstances [40–42]. In this study, we measured spatiotemporal parameters of gait during a simple walking task (straight walking at self-selected speed) and this could explain why we did not find differences between the two PD groups. Indeed, it has been previously reported that gait characteristics may be similar during simple gait tasks and differ during complex tasks between FOG+ and FOG- patients [43] mainly because of the nature of the gait task.

Finally, in FOG+ patients, we found a significant association between brain activity during AO of gait and the kinematic parameters recorded during normal walking task. Precisely, the activity at the level of the precuneus correlated with a worse performance in terms of step length and gait variability during normal walking. This association was not significant in PD FOG- patients and in healthy controls. This observation appears to fit with findings in HS where the activation of the precuneus was associated with the imagination of complex locomotor functions such as walking with obstacles [44], attention shift, and the processing of visuospatial stimulus [27]. Therefore, we could speculate that the activity at the level of the precuneus is crucial in the covert action of walking, in those subjects presenting walking difficulties, such as PD patients with FOG [45]. It would be interesting to investigate whether activity in this area might change with AO training.

In this study, there are several limitations we need to acknowledge. First, together with the relatively small sample size of the FOG-/FOG+ subgroups, we did not include a control condition in the "off" state. These are the main reasons for which we propose this article as a pilot investigation. However, this is the first study investigating neural correlates of AO of gait in a population of both FOG+ and FOG- PD patients, revealing that a functional reorganization occurs in PD patients and in particular in those presenting with FOG, which could be linked to a frontoparietal dysfunction. Furthermore, in the latter patients, the behavioral performance during gait is associated with activity at the parietal level (in particular, the precuneus) suggesting that these regions could have a role in AO rehabilitation of gait in FOG+ patients. Second, we did not monitor gaze movements during AO task in the MRI scan. Furthermore, while participants had time to familiarize with the task before the MRI, during the task-fMRI sequence, full adherence to the AO task during the MRI cannot be ensured. Third, gait parameters were recorded during usual walking task and via GAITRite®. Additional studies assessing gait during complex gait circumstances with wearable sensors are calls for better investigating possible relationship between cortical activations and gait features in FOG patients. Finally, further investigations are needed to better elucidate neural changes induced by AOT in PD and to exhibit if differences between patients with and without FOG exist.

Data Availability

The MRI and gait data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

EP reports grant from Michael J. Fox Foundation; EP and LA report honoraria for participating at Fox Trial Finder projects of the MJF Foundation; MI received research grants from NIH, DOD, NMSS, FISM, and Teva Neuroscience; RM, GB, MP, CC, and AB have no conflict of interest to report.

Authors' Contributions

Giulia Bommarito and Martina Putzolu equally contributed to this work.

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Supplementary Materials

Table S1: clusters of brain activation in PD patients (whole group) and HS and comparison between the two groups. Table S2: clusters of brain activation in FOG- and FOG+ patients. Table S3: subgroup comparison. Table S4: brain behavior correlation results. *(Supplementary Materials)*

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Review Article

Effects of Transcranial Direct Current Stimulation (tDCS) in the Normalization of Brain Activation in Patients with Neuropsychiatric Disorders: A Systematic Review of Neurophysiological and Neuroimaging Studies

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Background. People with neuropsychiatric disorders have been found to have abnormal brain activity, which is associated with the persistent functional impairment found in these patients. Recently, transcranial direct current stimulation (tDCS) has been shown to normalize this pathological brain activity, although the results are inconsistent. *Objective*. We explored whether tDCS alters and normalizes brain activity among patients with neuropsychiatric disorders. Moreover, we examined whether these changes in brain activity are clinically relevant, as evidenced by brain-behavior correlations. *Methods*. A systematic review was conducted according to PRISMA guidelines. Randomized controlled trials that studied the effects of tDCS on brain activity by comparing experimental and sham control groups using either electrophysiological or neuroimaging methods were included. *Results*. With convergent evidence from 16 neurophysiological/neuroimaging studies, active tDCS was shown to be able to induce changes in brain activation patterns in people with neuropsychiatric disorders. Importantly, anodal tDCS appeared to normalize aberrant brain activation in patients with schizophrenia and substance abuse, and the effect was selectively correlated with reaction times, task-specific accuracy performance, and some symptom severity measures. *Limitations and Conclusions*. Due to the inherent heterogeneity in brain activity measurements for tDCS studies among people with neuropsychiatric disorders, no meta-analysis was conducted. We recommend that future studies investigate the effect of repeated cathodal tDCS on brain activity. We suggest to clinicians that the prescription of 1-2 mA anodal stimulation for patients with schizophrenia may be a promising treatment to alleviate positive symptoms. This systematic review is registered with registration number CRD42020183608.

1. Introduction

Neuropsychiatric disorders, such as schizophrenia, depression, and substance abuse disorders, are a collection of mental health conditions that are characterized by behavioral, emotional, and cognitive disturbances, which significantly affect the social and occupational functioning of an individual [1]. Together, these diseases are the top contributor to the global burden of nonfatal disease, reportedly accounting for approximately 20% in 2016 [2], and this number is expected to increase further in the future [3]. Despite the marked differences in etiology, abnormal brain activity is a common manifestation shared among these disorders [4, 5].

Among the many indicators used in different methods of measurement, event-related potentials (ERP) [6, 7] and blood-oxygen-level-dependent (BOLD) signals [8, 9] are two of the most commonly adopted indicators of brain activity. Compared to healthy individuals, people with neuropsychiatric disorders exhibit distinctive patterns of brain activity when these two groups are presented with the same stimuli/-tasks that are believed to elicit task-relevant neural activation patterns. Regarding ERP, for example, people with schizophrenia have demonstrated consistently smaller P300 amplitudes than healthy individuals in various sustained attention tasks [10, 11] and the same has been shown in individuals with substance abuse disorders [12]; people with depression

showed a reversed pattern of P100 amplitude changes when processing happy and sad faces and an enhanced N170 in facial recognition [13]. In fMRI studies, people with neuropsychiatric disorders commonly exhibited abnormal activation in the prefrontal cortex during basic cognitive and executive functioning tasks, such as a reduction in dorsolateral prefrontal cortex activation in schizophrenia patients during working memory tasks [14], a reduction in inferior frontal gyrus activation in people with attention-deficit/hyperactivity disorder (ADHD) in attentional control tasks [15], and an increase in right medial frontal cortex activation in people with depression during tasks requiring attention and memory manipulation [16]. Given that such abnormalities are well documented to be associated with impaired cognitive [17], social [18], and emotional [19] functioning, clinicians and researchers have attempted to normalize the brain activity patterns of these patients through different treatment methods.

Pharmacological treatments, such as antidepressants and antipsychotics, are currently the most common way of promoting normalization of brain activities. An fMRI meta-analysis of nine studies showed that antidepressants restored prefrontal cortex hypoactivation and reduced limbic system hyperactivation in patients with depressive disorders [20], whereas the activation of the anterior cingulate cortex and insular cortex was found to be modulated by antipsychotics in people with psychosis [21]. However, these medications are often associated with undesirable side effects, such as extrapyramidal side effects induced by not only first- but also second-generation antipsychotics [22], as well as hyponatremia, bleeding, or seizures induced by serotonin reuptake inhibitors (SSRIs) [23], which hinder treatment compliance [24, 25]. Alternatively, transcranial direct current stimulation (tDCS), hypothesized to be able to normalize brain activation abnormalities in patients with neuropsychiatric diseases, has been rigorously studied recently in terms of its proposed effects. tDCS is a noninvasive neuromodulation technique that utilizes the delivery of a weak direct current (usually under 3 mA) [26] through the scalp to the brain with the use of oppositely charged electrodes (i.e., anode and cathode) to alter the brain areas underneath the electrodes [27]. Early studies in healthy individuals showed the promise of tDCS in modulating neuroplasticity [28] and cortical excitability [29] in healthy individuals, and this treatment was later found to be able to promote motor recovery in stroke patients by modulating the abnormal neural activation patterns resulting from stroke [30]. Recently, the effects of tDCS on the modulation of cognitive function have been increasingly studied in healthy individuals and have yielded positive results [30], and it has been shown that changes in brain activity after tDCS are associated with improved cognitive performance [31]. These findings further reinforce the potential of tDCS to become a promising treatment modality for people with neuropsychiatric disorders, who often exhibit cognition-related deficits.

Indeed, some studies have revealed that tDCS could normalize brain activation in patients with neurological/neuropsychiatric disorders [32, 33]. However, the results are inconsistent with negative results reported previously [34, 35]. Moreover, in order for tDCS to be developed as a clinically relevant treatment regimen, neural changes must be associated with clinical gains, yet studies that reported such a brain-behavior relationship also revealed divergent results (see [36] for positive results but [37] for negative results for tDCS treatment in people with the same neuropsychiatric diagnosis). In order to clarify the brain-behavior relationships, a systematic review of randomized controlled trials, comparing the neural effects of tDCS across studies, could help fill this knowledge gap; no such review, however, is currently available. To fill this gap, we aimed to determine (1) whether tDCS could induce changes in brain activation in patients with neuropsychiatric disorders, (2) whether it normalizes or worsens participants' outcomes, and (3) whether the neurophysiological effects are correlated with clinical/behavioral outcomes.

2. Methods

2.1. Literature Search. This systematic review was performed according to the PRISMA guidelines [38] and was registered in the International Prospective Register of Systematic Reviews (PROSPERO; register ID CRD42020183608). A systematic literature search was carried out in March 2020 with the search terms "transcranial direct current stimulation", "tDCS", "functional magnetic resonance imaging", "fMRI", "electroencephalography", and "EEG" in the electronic databases PubMed, Scopus, and Embase using title, abstract, and keyword searches (see Supplementary Materials for the actual search strategies for each of the databases). An additional search was performed one month before the submission (i.e., 20 June 2020) to ensure that all retrievable records were included. No limit was set on the publication dates. We also manually searched the bibliographies of related studies to identify possible articles to be included in this review.

2.2. Study Inclusion. Randomized controlled trials with tDCS administration on patients with neuropsychiatric disorders as defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [1] with the ERP/brain blood flow outcome measured by EEG/fMRI were included in this review. We conducted three stages of screening to identify suitable records for inclusion in the systematic review. Duplicate records were first removed, after which we screened the titles and abstracts of the remaining articles to exclude studies without peer-reviewed empirical data (e.g., reviews, conference proceedings, book chapters, and editorials), nonhuman studies, studies that did not apply tDCS on patients with any type of neuropsychiatric disorder, studies that did not apply tDCS as the sole brain stimulation technique, studies where no EEG/fMRI measures were adopted, and studies without English full text. The third step was full-text screening of the remaining studies, which was conducted to exclude non-randomized studies, studies without a sham tDCS control group, studies not measuring and presenting results regarding ERP and blood flow changes before and after tDCS, and studies that did not give between-group (i.e., active versus sham) comparisons that reflected tDCS effects. Two

personnel (i.e., two research assistants: K.C. and A.C.) conducted the above screening separately. The second author resolved any discrepancies between the decisions made and provided the final judgement regarding the inclusion of studies.

2.3. Data Extraction. Two research assistants (P.H. and E.L.) extracted the demographic details (i.e., the numbers of participants in the sham and active tDCS groups as well as the participants' ages, psychiatric diagnoses, and medication status), tDCS protocol details (i.e., mode of stimulation, electrode size and montage, duration of stimulation, stimulation intensity, therapy/task accompanied by tDCS delivery, and relevant details), and outcome measures (i.e., experimental paradigm for recording ERP/cerebral blood flow, primary behavioral/clinical outcome results, and correlation between brain activity changes and clinical outcome). Information discrepancies in data extraction were confirmed and resolved by the first author. Electronic mails were sent to corresponding authors to ask for additional information/-clarification if the data to be extracted were not complete.

2.4. Data Synthesis and Analysis. To determine whether tDCS outcomes from individual studies were appropriate to be pooled with meta-analytic techniques, we subjectively evaluate the clinical heterogeneity of patients, interventions, and outcomes, as well as the methodological heterogeneity in study design in all included studies; as recommended by Rao et al. [39], meta-analysis would not be conducted if either or both forms of heterogeneity were judged to be substantial. To address the question of whether tDCS induces changes in brain activation patterns in people with neuropsychiatric disorders, we provide an overall narrative synthesis of results. In order to address whether tDCS could normalize brain activation for different neuropsychiatric disorders, we first conducted a brief review of a previous meta-analysis regarding the abnormalities of brain activation in patients compared to healthy controls, such that we could determine whether the brain activity change induced by tDCS could be said to be a "normalization." In order to explore whether the normalization effects brought by tDCS underlie behavioral/-clinical improvements, narrative synthesis was conducted to summarize the brain-behavior relationship data reported in each of the included studies. If meta-analysis was deemed appropriate, effect size calculation and generation of the forest plot would be performed using Comprehensive Meta-Analysis (CMA; Biostat, Englewood, NJ) software; when test statistics could not be obtained from the corresponding authors but the results were described in text, nonsignificant and significant results would be assumed to have p values of 0.5 (1-tailed) and 0.05 [40], respectively. The risk of bias in individual studies was assessed by using the Cochrane Collaboration's tool [41] which was conducted by the first author and a research assistant (M. Cheng).

3. Results

3.1. Study Selection. A total of 16 studies (with 22 experiments) were included in this review. The electronic database search yielded a total of 1968 studies, with 1005 records remaining for abstract screening after the removal of 963 duplicated records. 880 studies were excluded after exclusion criteria were applied at this stage. The full text of 132 records was further assessed for inclusion in the systematic review. A total of 109 studies were further excluded with additional exclusion criteria applied. See Figure 1 for the diagram illustrating the article screening procedure.

3.2. Study Characteristics. All of the experiments adopted prefrontal montage, except for experiments with temporal montage (experiments 1 and 2 from Rahimi et al. [42], experiment 1 from Impey et al. [43]) and one experiment investigating the effects of parietal montage (experiment 1 from Kim et al. [35]). The treatment duration for each session was 20 minutes for all studies except 15 minutes in den Uyl et al. [44] and 30 minutes in Orlov et al. [45]. Nine studies measured ERP, while the remaining seven studies investigated changes measured by fMRI. Seven studies investigated the effects of tDCS on brain activation in individuals with schizophrenia, and all of these studies involved patients with illness onset more than ten years with an average of 18.6 years [35, 43, 45–49]. Three studies investigated the effects of tDCS in people with substance abuse disorders [44, 50, 51]. One study investigated the effects of tDCS in individuals with depression [52]. A total of three studies investigated the effects of tDCS on neurodevelopmental disorders, with two on ADHD [53, 54] and one on dyslexia [42]. Two studies investigated MCI [33, 55]. The demographic details, tDCS protocols, clinical/behavioral outcomes, and brain-behavior relationship results are listed in Table 1. In view of the substantial clinical and methodological heterogeneity observed across the included papers, no meta-analysis was performed.

3.3. Risk of Bias. With reference to Figure 2, more than half of the studies adopted adequate blinding procedures during treatment administration and reported all data from planned analysis to prevent reporting bias; for crossover studies, most of the studies adopted a washout period of more than two days to prevent carryover effects. However, most studies showed unclear bias in terms of random sequence generation, allocation concealment, blinding of outcome assessment, and incomplete outcome data. Figure 2(a) displays the risk of bias items presented as percentages across studies, and Figure 2(b) shows the risk of bias summary for each included study.

3.4. Can tDCS Induce Changes in Brain Activation Patterns in People with Neuropsychiatric Disorders?

3.4.1. ERP Studies. Five studies reported the effects of tDCS in modulating P300 amplitude [44, 46, 48, 51, 54]. All of these studies applied prefrontal stimulation (stimulating electrode placed over DLPFC, IFG, and supraorbital regions). Anodal stimulation was investigated in all of these studies, while the effects of cathodal stimulation were also studied in Dunn et al. [48] and Rassovsky et al. [46]. Overall, anodal tDCS was able to normalize P300 amplitude across these studies, while the effects of cathodal stimulation remained inconclusive. Three studies reported MMN amplitude changes [43, 46,

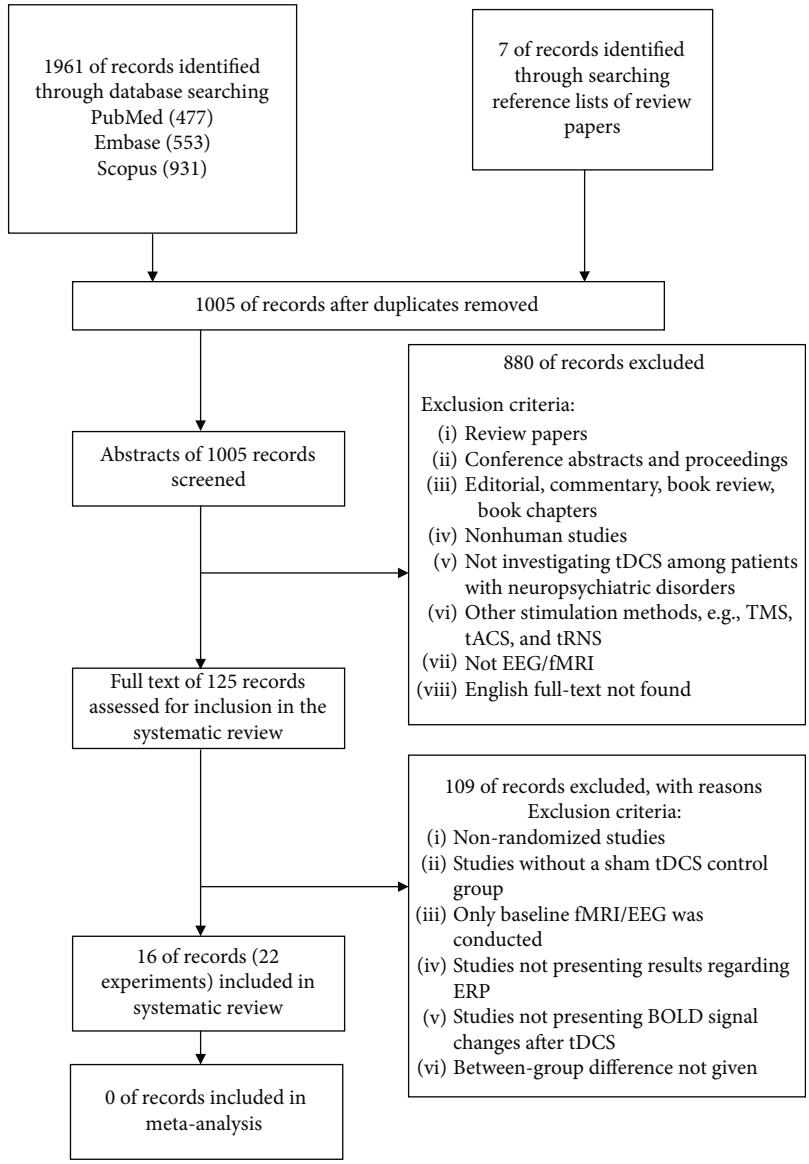


Figure 1: A flowchart illustrating the article screening process.

48]. Two studies adopted the prefrontal (DLPFC and supraorbital regions) montage, and the remaining study adopted the temporal montage [43]. Anodal stimulation was shown to reduce MMN amplitude, while cathodal stimulation remains inconclusive. Three experiments reported changes in N100 amplitude for anodal [42, 49]and bilateral [42] stimulation, showing that N100 was normalized by both stimulation modes, while Rahimi et al. [42] also reported a significant increase in P100 and P200 amplitude after either anodal or bilateral tDCS over the temporal region. Finally, anodal stimulation was also found to reduce the amplitude of N200 [54] but not for N170 [46], but cathodal stimulation could enhance the amplitude of N170 as stated in Rassovsky et al. [46].

3.4.2. fMRI Studies. Seven studies investigated BOLD signal changes at the whole-brain level/*a priori* ROI after anodal tDCS over the prefrontal cortex when compared to sham-stimulated controls [33, 45, 50, 52, 53]. These experiments collectively suggested that anodal stimulation could increase BOLD signals not only over the brain regions directly under the stimulating electrode but also in regions remote from the expected stimulated areas. The remaining two studies reported between-group differences in changes in interhemispheric imbalance [35] and regional cerebral blood flow (rCBF) after active and sham tDCS, respectively. Kim et al. [35] reported that bilateral stimulation significantly normalized the interhemispheric imbalance in the active anodal stimulation group when compared to sham-stimulated individuals, while Das et al. [55] revealed an increase in rCBF in the right medial prefrontal cortex at rest after applying anodal stimulation over the left IFG.

3.5. Can tDCS Normalize Brain Activation in Different Patients with Different Neuropsychiatric Diagnoses? A review of previous meta-analyses showing the aberrant brain

Table 1: Summary of included studies.

Study [first author (year)]	Patient characteristics				Experimental details					Outcome measures		
	Diagnosis	Total *N*	Age group	Concurrent mediation	Mode[†]	Montage[‡]	Duration (min)	Total number of sessions	Intensity (mA)	Therapy/task accompanied by tDCS	Modality (EEG/fMRI): paradigm	Behavioral/clinical findings ($\alpha = 0.05$; active vs. sham)	Brain-behavior relationship ($\alpha = 0.05$)
Schizophrenia													
Kim (2019) [35]	Schizophrenia	11	Adult	Yes	Expt 1: bilateral	Anode: P4 Cathode: P3	20	1	2	Nil	fMRI: illness awareness task	fMRI illness awareness task (i) Level of insight: n.s.	Correlations between interhemispheric imbalance in CBF and clinical scores: n.s.
		11			Expt 2: bilateral	Anode: F4 Cathode: F3	30	1	2	Nil	fMRI: illness awareness task		
Orlov (2017) [45]	Schizophrenia	24	Adult	Yes	Anodal	Anode: F3 Cathode: Fp2	30	1	2	Working memory task (*n*-back)	fMRI: working memory task (*n*-back)	fMRI *n*-back task (i) RT: n.s.	Significant correlation between the 2-/3-back performance 1 day after tDCS and the increased activation in L DLPFC ($p < 0.05$)
										Stroop task	fMRI: stroop task	fMRI stroop task (i) ACC: n.s.	Significant correlation between accuracy performance in the incongruent condition activation in the ACC ($p < 0.005$)
Rassovsky (2018) [46]	Schizophrenia	37	Adult	Yes	Expt 1: anodal	Anode: F3 Cathode: Fp2	20	2	2	Nil	EEG: auditory oddball task EEG: emotion recognition task	Clinical assessment performance (ACC) (i) Speed of processing: n.s. (ii) Working memory: n.s. (iii) Verbal memory: n.s. Reasoning and problem solving: n.s.	Correlation between EEG results from the active group and cognitive scores: n.s.
		37			Expt 2: cathodal	Cathode: F3 Anode: Fp2	20	2	2	Nil	EEG: auditory oddball task EEG: Emotion recognition task		
Reinhart (2015) [47]	Schizophrenia	17	Adult	Yes	Anodal	Anode: FCz Cathode: R cheek	20	1	1.5	Nil	EEG: feedback-based learning task	EEG feedback-based learning task (i) RT: n.s.	Gain in ERN amplitude correlated with lower delusion score ($p < 0.0001$)
Dunn (2016) [48]	Schizophrenia	24	Adult	Yes	Expt 1: anodal	Anode: Fp1, Fp2 Cathode: R upper arm	20	2	1	Nil	EEG: auditory oddball task EEG: passive attention auditory	Not stated	Not stated

Table 1: Continued.

Study [first author (year)]	Patient characteristics				Experimental details					Therapy/task accompanied by tDCS	Outcome measures		
	Diagnosis	Total *N*	Age group	Concurrent mediation	Mode[†]	Montage[‡]	Duration (min)	Total number of sessions	Intensity (mA)		Modality (EEG/fMRI): paradigm	Behavioral/clinical findings ($\alpha = 0.05$; active vs. sham)	Brain-behavior relationship ($\alpha = 0$ .05)
											duration deviant paradigm EEG: auditory oddball task		
		24			Expt 2: cathodal	Cathode: Fp1, Fp2 Anode: R upper arm	20	2	1	Nil	EEG: passive attention auditory duration deviant paradigm		
		12			Expt 1: anodal	Anode: between C5 and T7 Cathodal: Fp2	20	1	2	Nil		Behavioral working memory task (2-back) (i) RT: n.s. (ii) ACC: n.s.	Not stated
Impey (2017) [43]	Schizophrenia		Adult	Yes							EEG: auditory oddball task		
		12			Expt 2: anodal	Anode: F3 Cathode: Fp2	20	1	2	Nil		Behavioral working memory task (2-back) (i) RT: $p < 0.05$ (active < sham) (ii) ACC: $p < 0.05$ (active > sham)	Greater frontal MMN change correlated with faster RT ($p < 0.05$)
Knechtel (2014) [49]	Schizophrenia	14	Adult	Yes	Anodal	Anode: F3 Cathode: Fp2	20	1	2	Nil	EEG: go/no-go task	EEG go/no-go task (i) ACC: n.s.	Not stated
Substance abuse													
Mondino (2018) [50]	Tobacco abuse	24	Adult	No	Anodal	Anode: between F4 and Fp2 Cathode: between O1 and T5	20	10	1	Nil	fMRI: visual oddball task (smoking-related vs. neutral)	Reduction in craving: $p < 0.05$ (active > sham)	R PCC increase in activation does not correlate with changes in cigarette consumption: n.s.
den Uyl (2016) [44]	Alcohol abuse	39	Adult	No	Anodal	Anode: F3 Cathode: Fp2	15	3	1	Nil	EEG: visual oddball task (alcohol-related image vs. neutral image)	Reduction in craving: n.s.	Not stated
Conti (2014) [51]	Crack-cocaine abuse	13	Adult	No	Bilateral	Anode: F4 Cathode: F3	20	1	1	Nil	EEG: visual oddball task (crack-related image	Not stated	Not stated

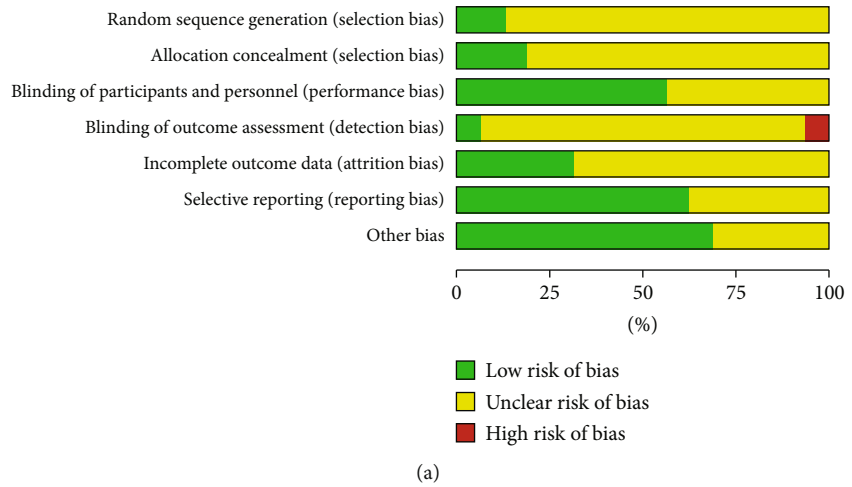
Table 1: Continued.

Study [first author (year)]	Patient characteristics				Experimental details					Therapy/task accompanied by tDCS	Outcome measures		
	Diagnosis	Total *N*	Age group	Concurrent mediation	Mode[†]	Montage[‡]	Duration (min)	Total number of sessions	Intensity (mA)		Modality (EEG/fMRI): paradigm	Behavioral/clinical findings ($\alpha = 0.05$; active vs. sham)	Brain-behavior relationship ($\alpha = 0$ .05)
											vs. neutral image)		
Depression													
Nord (2019) [52]	Depression	39	Adult	No	Anodal	Anode: F3 Cathode: L deltoid	20	8	1	60-minute cognitive behavioral therapy with senior CP (symptom relief)	fMRI: working memory task (*n*-back) fMRI: emotional processing task	fMRI *n*-back task: (i) RT: n.s. (ii) ACC: n.s. (i) fMRI emotional processing task (ii) ACC: n.s.	Correlation between increased bilateral DLPFC activation and *n*-back improvement: n.s. Correlation between amygdala activation and emotional processing task: n.s.
Neurodevelopmental disorders													
Sotnikova (2017) [53]	Attention deficit/hyperactivity disorder	16	Adolescent	No	Anodal	Anode: F3 Cathode: Cz	20	1	1	Working memory task (*n*-back)	fMRI: working memory task (*n*-back)	fMRI *n*-back task ACC: <0.05 (active > sham)	Not stated
Breitling (2020) [54]	Attention deficit/hyperactivity disorder	15	Adolescent	No	Expt 1: anodal	Anode: F8 Cathode: Fp1	20	1	1	Working memory task (*n*-back)	EEG: working memory (*n* -back)	EEG *n*-back task (i) ACC: n.s. (ii) RT: n.s.	Not stated
		15			Expt 1: anodal (HD-tDCS)	Anode: F8 with 4 surrounding cathodes	20	1	0.5	Working memory task (*n*-back)		EEG *n*-back task (i) ACC: n.s. (ii) RT: n.s.	
Rahimi (2019) [42]	Dyslexia	17	Children	No	Expt 1: anodal	Anode: T7 Cathode: R shoulder	20	1	1	Nil	EEG: gap detection task	(i) EEG gap detection task temporal perception: $p < 0.001$ (active > sham)	Not stated
		17			Expt 2: bilateral	Anode: T7 Cathode: T8	20	1	1	Nil		(i) EEG gap detection task ACC: $p < 0.001$ (active > sham)	
Neurodegenerative disorders													
Das (2019) [55]	Mild cognitive impairment	16	Elderly	No	Anodal	Anode: F7 Cathode: R shoulder	20	8	2	Reasoning and inferencing strategy training	fMRI: resting	Clinical assessment performance (ACC) (i) Task switching: $p < 0.05$ (active > sham) (ii) Strategic learning: $p < 0.05$	Correlation between regional CBF and clinical improvement: n.s.

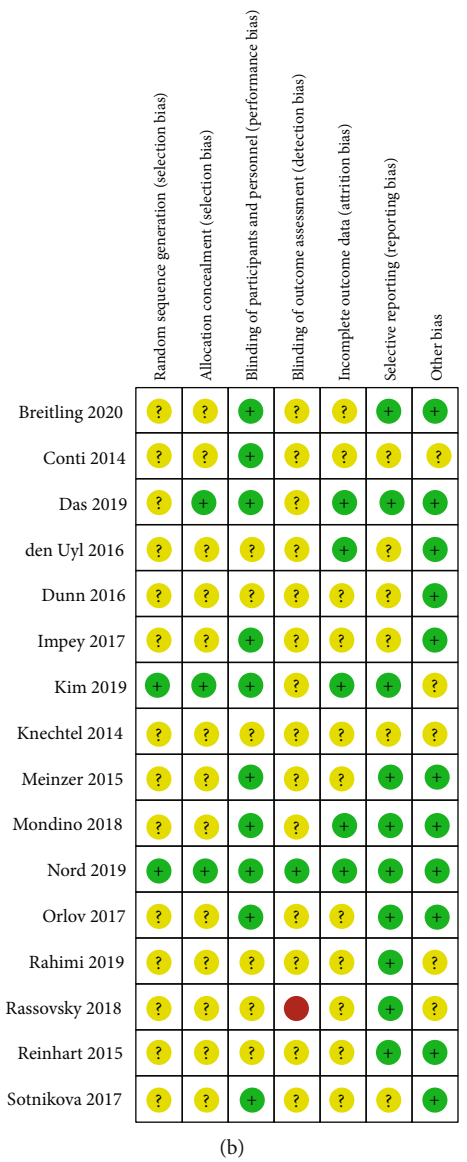
Table 1: Continued.

Study [first author (year)]	Patient characteristics				Experimental details						Outcome measures		
	Diagnosis	Total *N*	Age group	Concurrent mediation	Mode[†]	Montage[‡]	Duration (min)	Total number of sessions	Intensity (mA)	Therapy/task accompanied by tDCS	Modality (EEG/fMRI): paradigm	Behavioral/clinical findings ($\alpha = 0.05$; active vs. sham)	Brain-behavior relationship ($\alpha = 0.05$)
Meinzer (2015) [33]	Mild cognitive impairment	18	Elderly	No	Anodal	Anode: F7 Cathode: Fp2	20	1	1	Semantic memory task	fMRI semantic word retrieval task	(active > sham) (iii) Episodic memory: $p < 0.05$ (active > sham) fMRI semantic word retrieval task (i) ACC: $p < 0.05$ (active > sham)	Correlation between changes in activation and reduction in errors: n.s.

[†]Mode of stimulation was classified based on a previously published framework [56]. [‡]Montage location was reported according to the EEG 10-20 system; the anatomical positions were reported for extracephalic montage. Expt: experiment; *N*: number of participants; EEG: electroencephalography; fMRI: functional magnetic resonance imaging; L: left; R: right; n.s.: nonsignificant (at $\alpha = 0.05$ significance level); RT: reaction time; ACC: accuracy; ACC: anterior cingulate gyrus; PCC: posterior cingulate cortex; DLPFC: dorsolateral prefrontal cortex; ERN: event-related negativity; MMN: mismatch negativity; HD-tDCS: high-definition transcranial direct current stimulation; CBF: cerebral blood flow.



(a)



(b)

Figure 2: (a) A chart presenting authors' judgement as percentage about each risk of bias item across all included studies. (b) A chart showing authors' judgements about each risk of bias item for each included study.

activation patterns in patients with neuropsychiatric disorders included in this study is presented in Table 2.

3.5.1. Schizophrenia. Among the 11 experiments, while seven experiments investigated anodal tDCS effects, two studies investigated cathodal and the remaining two investigated bilateral tDCS effects. With reference to previous meta-analyses and empirical studies, patients with schizophrenia were found to have reduced P300 [11, 57], N170 [58], N100 [59], MMN [60, 61], and ERN [62, 63] amplitudes when compared to healthy controls. Active anodal as well as bilateral tDCS stimulations were found to enhance the amplitudes of these ERP components [43, 46–49]. fMRI meta-analysis reviewed that while the medial frontal cortex was shown to have reduced activation during working memory tasks, the anterior cingulate cortex (ACC) was found to be hyperactivated during attentional control tasks in people with schizophrenia [14]; this phenomenon was also shown to be reversed by anodal tDCS [45]. For cathodal tDCS, the normalization effects remained inconclusive [46, 48].

3.5.2. Substance Abuse. All three studies applied anodal stimulation. A previous meta-analysis showed that patients with substance abuse were found to have reduced P300 amplitude during auditory oddball tasks [12] and bilateral PCC activation reduction [64] at rest, which was found to be significantly enhanced after the application of anodal tDCS when compared to the sham control tDCS group [44, 50, 51].

3.5.3. Depression. It was found that the left DLPFC was hypoactive in patients with depression, as reflected in a previous meta-analysis [65]. The sole study [52] investigating anodal tDCS in modulating brain activation for working memory and emotional face processing demonstrated that while there were no significant differences in DLPFC activation changes before and after the treatment between sham and active tDCS for working memory tasks, increased left DLPFC activation during the emotional face processing task reflected the normalization of brain activation for these patients.

3.5.4. Neurodevelopmental Disorders. Previous meta-analyses revealed that ADHD patients showed reduced P300 amplitude [66], reduced DLPFC, SMA, PMC [67], and insula [68] activation, and enhanced precuneus [68] activation compared to their healthy counterparts. Anodal tDCS was shown to normalize aberrant brain activity, except for the enhancement of precuneus activation, which has already been shown to be enhanced in ADHD [53]. P300 was found to be enhanced regardless of the use of conventional or HD-tDCS, with the magnitude of enhancement being larger in HD-tDCS, although the difference in magnitude does not reach statistical significance [54]. Regarding dyslexia, other empirical studies except Rahimi et al. [42] have identified P100 [69] and N100 [70, 71] amplitude abnormalities, although the direction of effects remained inconclusive, as no meta-analysis could be identified. After anodal tDCS, it was found that P100, N100, and P200 amplitudes were reduced, although it remains debatable whether these changes reflect normalization.

3.5.5. Neurodegenerative Disorders. Although meta-analyses were not available, two reviews reported a decrease in resting cerebral blood flow (CBF) and reduced activation in the inferior frontal gyrus in patients with MCI compared to healthy individuals. Anodal tDCS was found to enhance prefrontal CBF [55], reflecting a normalization effect, but it was also found to reduce activation in the bilateral IFG [33], which ran counter to normalization.

3.6. Brain-Behavior Relationship. Eight of the 16 included studies reported results of the correlations between changes in brain activity and behavioral/clinical outcomes after tDCS. When reaction time (RT) performances in memory [45] and learning [43] tasks were investigated as a behavioral indicator, significant correlations between reduction in RT with increased activation in the left dorsolateral prefrontal cortex (DLPFC) and increased frontal mismatch negativity (MMN) amplitude were reported. For on-task accuracy performance, although eight experiments reported between-group differences, only three experiments reported brain-behavior correlations; in Orlov et al. [45], the same attentional control task (i.e., the Stroop task) was given during tDCS stimulation and pre-/post-tDCS assessments and increased activation in the anterior cingulate cortex (ACC) significantly correlated with accuracy improvement before and after tDCS. For other experiments in which the assessment and treatment tasks were nonidentical, nonsignificant correlations were found between accuracy results and changes in activation in the anterior cingulate cortex for an untrained semantic memory retrieval task [33], as well as in working memory task [52]. When the relationship between psychiatric symptom changes and brain activity after tDCS was studied, Reinhart et al. [47] reported significant correlations between an increase in error-related negativity (ERN) and a reduction in the severity of delusional symptoms, while the correlation between changes in the severity of depressive symptoms and DLPFC/ACC activation [52], as well as the relationship between changes in the frequency of addictive behaviors and the right posterior cingulate gyrus (PCC) [50], was nonsignificant. Three studies investigated the correlations between score changes in standardized neurocognitive [46, 55], sociocognitive [46], and metacognitive [35] assessments with brain activity, and nonsignificant relationships were reported for all of these experiments.

4. Discussion

This systematic review was aimed at investigating the effects of tDCS in normalizing aberrant brain activities among people with neuropsychiatric disorders. After conducting a comprehensive literature search by browsing electronic databases and manual searches from the reference lists of relevant studies, 16 studies with 22 experiments that studied tDCS effects with ERP or fMRI activation measures were included in this systematic review. With converging evidence from both neurophysiological and neuroimaging studies, tDCS was shown to be able to induce changes in brain activation patterns in people with neuropsychiatric disorders. Importantly, anodal tDCS appeared to normalize aberrant brain activation in

Table 2: Abnormal brain activation of patients with neuropsychiatric disorders when compared to healthy controls.

Diagnosis	EEG/fMRI indicator	Task	Increase/decrease when compared to controls	Meta-analytic reference (if applicable)
Schizophrenia	P300	Auditory oddball	Decrease	Bramon et al. (2004) Qiu et al. (2014)
	N170	Face processing	Decrease	McCleery et al. (2015)
	N100	Paired click paradigm	Decrease	Rosburg (2018)
	ERN†	Attentional control	Decrease	Foti et al. (2012) Mathalon & Ford (2012)
	MMN	Auditory discrimination	Decrease	Umbricht & Krlijes (2005) Erikson et al. (2016)
	MFC activation	Working memory	Decrease	Glahn et al. (2005)
	ACC activation	Attentional control	Increase	Glahn et al. (2005)
Substance abuse	P300	Auditory oddball	Decrease	Euser et al. (2012)
	PCC activation	Resting	Decrease	Xiao et al. (2015)
Depression	L DLPFC activation	Working memory, emotional face processing	Decrease	Groenewold et al. (2013)
Attention deficit/hyperactivity disorder	P300	Auditory oddball	Decrease	Szuromi et al. (2011)
	DLPFC activation	Working memory, attention	Decrease	Cortese et al. (2012)
	SMA activation		Decrease	
	PMC activation		Decrease	
	Insula activation		Decrease	Hart et al. (2012)
	Precuneus activation		Increase	
Dyslexia†	P100	Auditory	Increase	Rahimi et al. (2019) Araujo et al. (2015), right brain
	N100		Increase	Rahimi et al. (2019) Helenius et al. (2002)
			Decrease	Bonte & Blomert (2004)
	P200		Increase	Rahimi et al. (2019)
Mild cognitive impairment‡	Prefrontal resting CBF	Resting	Decrease	Hays et al. (2016)
	IFG activation	Semantic memory retrieval	Decrease	Nellessen et al. (2014)

†Meta-analysis/review not available; results from empirical studies were reported. ‡Meta-analysis not available; results from systematic review were reported. EEG: electroencephalography; fMRI: functional magnetic resonance imaging; ERN: event-related negativity; MMN: mismatch negativity; MFC: medial frontal cortex; ACC: anterior cingulate gyrus; PCC: posterior cingulate cortex; L: left; DLPFC: dorsolateral prefrontal cortex; SMA: supplementary motor area; PMC: premotor cortex; IFG: inferior frontal gyrus; CBF: cerebral blood flow.

patients with schizophrenia and substance abuse, with this effect being selectively correlated with reaction times, task-specific accuracy performance, and some symptom severity measures. We first discuss the normalization effects and treatment implications in schizophrenia and other neuropsychiatric disorders, followed by an account regarding the phenomenon observed for the brain-behavior relationship.

4.1. Brain Activity Normalization in Schizophrenia and Other Psychiatric Diagnoses: Treatment Implications and Possible Research Development. Across all psychiatric diagnoses, the brain activity normalization effects of tDCS were most studied in patients with schizophrenia. In particular, prefrontal tDCS showed the most evidence of normalizing brain activity across different ERP and fMRI parameters that were identified to be aberrant in these patients in previously published meta-analytic data. Notably, these results can be generalized only to patients with chronic schizophrenia, given that the included population had mean illness duration of 18.6 years. Although the brain normalization effect was statistically significant, the majority of accuracy and reaction time performance in cognitive tasks showed nonsignificant improvements after the treatment. This was consistent with the nonsignificant behavioral findings reported by a meta-analysis of single-session tDCS in healthy individuals [72]. There are three possible reasons to explain this. First, and probably the most common problem existing in the current tDCS literature on patients with neuropsychiatric disorders, is the lack of power of the included studies to detect behavioral changes. Second, previous behavioral research

suggested that there are interindividual variabilities in response to tDCS; while some might benefit from tDCS, some participants might actually show impaired cognitive performance after tDCS [73–75]. Indeed, another study included in this review by Nord et al. [52] reported that there are neural predictors that determine the behavioral treatment outcome; for instance, they reported that pretreatment activation of the left DLPFC was positively associated with posttreatment depressive symptom improvement. Collectively, these results imply that tDCS might be suitable only for some of the patients to optimize treatment gain and the decision of who would benefit and who would not depend on our understanding of the neural predictors, which is currently in the very early stages of research. Third, the lack of pairing with a cognitive task (e.g., working memory training) during tDCS delivery might contribute to the nonsignificant behavioral gains despite the significant neural gains. A previous meta-analysis has shown that concurrent working memory training could promote a small but significant effect of DLPFC anodal stimulation [76]. However, what kind of task should be administered and how it should be administered are some of the key questions to be studied, especially for cognitive enhancement with tDCS, given that previous reports have shown that anodal tDCS *per se* could facilitate or inhibit cortical excitability, which depended solely on the speed of the motor task being performed [77].

Regarding the neural effects of tDCS on other psychiatric diagnoses (i.e., substance abuse disorders, ADHD, dyslexia, depression, and MCI), we observed that tDCS tends to demonstrate the normalization effects as well, but more studies have to be performed regarding each of the individual diagnosis to yield conclusive results; additionally, for some diagnoses in which the abnormal brain activity is still under debate (e.g., inconclusive results in N100 amplitude between patients with dyslexia and healthy controls [42, 70, 71]), tDCS might be regarded as a tool to probe neural activity [78] and enhance our understanding of these diseases in the future, rather than as a treatment.

4.2. Selective Correlation of Brain Normalization with Behavioral/Clinical Measures. It appears that, in the first place, the brain activity changes were not significantly correlated with behavioral/clinical outcomes in the majority proportion of studies, making it hard to see the clinical relevance of tDCS, which is considered one of the prerequisites for introducing tDCS to become a promising treatment regimen in daily clinical practice. However, it was observed from this review that only some indicators reflecting brain activity changes correlate with particular parameters, namely, the correlation of reaction times with MMN and left DLPFC activation during memory and learning tasks, accuracy rates of trained tasks administered during tDCS stimulation, and ERN amplitude changes with particular disease severity measures. We examined this phenomenon by understanding the possible neuronal mechanisms of tDCS. Increasing direct evidence from animal studies has shown that tDCS could moderate NMDAR-dependent synaptic plasticity (see Cavaleiro et al. [79] for a review), and human magnetic resonance spectroscopy (MRS) studies showed that tDCS could modulate the concentration of gamma-aminobutyric acid (GABA), a neurotransmitter acting at inhibitory synapses in the brain [80]. This translational evidence leads to the hypothesis that tDCS might bring about specific behavioral changes by moderating synaptic plasticity of the stimulated brain regions as well as the functionally connected networks [81]. Given the established relationships between (1) GABA and reaction time [82], (2) GABA and MMN [83], and (3) MMN and synaptic plasticity [84, 85], we could interpret the significant brain-behavior relationships between RT and brain activation changes in [43, 45] as indirect evidence showing the effects of tDCS in modulating synaptic plasticity. Following the above proposition, when tDCS stimulation was directly applied to the core brain regions underlying a specific psychiatric symptom, e.g., delusion, a psychiatric symptom that has been recently found to be underlain by the deficits of the cognitive control circuit with ACC being the core neural correlate [86], significant brain-behavior relationships could be expected using the appropriate biomarker (i.e., ERN has been recognized as an electrophysiological index of ACC activation [87–89]) to reflect brain activity changes and a sensitive assessment tool that reflects clinical changes, as documented by Reinhart et al. [47].

On the other hand, the relationships between tDCS-induced brain activity changes, accuracy, and other standardized cognitive measures appeared to be mediated by the presence of the highly specific task during stimulation. Many empirical studies have demonstrated the task-specific effects of tDCS aimed at enhancing memory [90], learning [91], and other higher-order cognitive functions [92, 93]. From computational modeling [94] and animal studies [95, 96], it has been shown that the electric field induced by tDCS is low (below 1 V/m) at a stimulation intensity between 1 and 2 mA, resulting in "subthreshold" (rather than "suprathreshold" stimulation applied by transcranial magnetic stimulation) neuromodulatory effects over ongoing neural processes. In other words, tDCS preferentially modulates the task-activated network; without the concurrent tasks guiding the stimulation effects, tDCS might not recruit the targeted network, for example, the aberrant neural network associated with various types of neuropsychiatric illness. Indeed, when we compared the significant correlation between accuracy performance in the emotional attentional control (Stroop) task and ACC resulting from presenting the same experimental paradigm before, during, and after tDCS as reported by Orlov et al. [45], with other studies given nonidentical training during tDCS administration [33, 52], it might be possible that due to the recruitment of different networks during pre-/post-treatment EEG/fMRI assessments when compared to the brain network recruited during therapy sessions, brain-behavior correlations could not be established. This would bring about another issue: does it mean the transfer of tDCS cognitive enhancement effects might be very limited, given that only highly specific tasks induce brain changes that are correlated with behavioral changes? In fact, previous research has demonstrated the potential of repetitive, task-relevant tDCS administered on consecutive days to promote cognitive skill transfer, which can last for

nine months among healthy subjects [90]. Although studies that applied repetitive tDCS in our current review did not show significant correlations with accuracy and scores from standardized cognitive assessments [52, 55] given the small sample size of each study and the limited number of repetitive tDCS studies available, future research might further investigate the longitudinal effects of repetitive tDCS in establishing brain-behavior relationships, an increasingly studied issue that potentially supports tDCS as a clinically relevant option for neurorehabilitation.

5. Limitations

Although we planned to conduct a meta-analysis for each separate neuropsychiatric diagnosis if the number of articles met the *a priori* threshold set by the power analysis, such analysis was not conducted due to the limited number of studies available; instead, only systematic review was conducted to address our enquiry. In addition, the exclusion of non-English articles and data published in other publication genres (e.g., conference abstracts, letters and commentaries, and thesis) might limit the generalizability of our review. Furthermore, it should be noted that the majority of papers included in this review did not explicitly report the procedures for random sequence generation, allocation concealment, and the blinding of assessors; hence, selection and detection biases might be induced and influenced the validity of results. Regarding the data availability, although we have contacted the corresponding authors for the studies that provide insufficient information for our analyses, we did not receive their reply before the data analysis, or even before this manuscript is submitted. Furthermore, we found that brain-behavior correlations were not reported in seven studies, and our analysis of this relationship could be based only on the available significant and nonsignificant results. Future studies might consider the investigation of brain-behavior correlations such that the clinical relevance of tDCS application could be further understood, which could in turn benefit the development of novel treatments for patients with neuropsychiatric disorders.

6. Conclusion

This systematic review was aimed at investigating the effects of tDCS in normalizing aberrant brain activities among people with neuropsychiatric disorders as well as the clinical relevance of tDCS regarding its effects in moderating brain activations. With convergent evidence from both neurophysiological and neuroimaging studies, tDCS was shown to be able to induce changes in brain activation patterns in people with neuropsychiatric disorders. Anodal tDCS appeared to normalize aberrant brain activation in patients with some psychiatric diagnoses, namely, schizophrenia and substance abuse disorders. The detection of brain-behavior correlations in some specific measures but not others might imply a need for careful consideration of the choice of behavioral measurements, as well as therapy/task design that engages the appropriate cognitive neuronal networks, to improve the clinical relevance of tDCS. Such improvements will be an important factor determining the fate of tDCS in neuropsychiatric practice in the future.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

M.C. was responsible for designing the study, conducting data analysis, interpreting the results, and writing the manuscript. Y.H. was responsible for the conception of the study, assisting in data analysis, interpreting the results, and revising the manuscript. All authors read and approved the final manuscript.

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Supplementary Materials

Literature search strategies applied for different electronic databases in this review. *(Supplementary Materials)*

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Research Article

EEG Correlates of Central Origin of Cancer-Related Fatigue

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The neurophysiological mechanism of cancer-related fatigue (CRF) remains poorly understood. EEG was examined during a sustained submaximal contraction (SC) task to further understand our prior research findings of greater central contribution to early fatigue during SC in CRF. Advanced cancer patients and matched healthy controls performed an elbow flexor SC until task failure while undergoing neuromuscular testing and EEG recording. EEG power changes over left and right sensorimotor cortices were analyzed and correlated with brief fatigue inventory (BFI) score and evoked muscle force, a measure of central fatigue. Brain electrical activity changes during the SC differed in CRF from healthy subjects mainly in the *theta* (4-8 Hz) and *beta* (12-30 Hz) bands in the contralateral (to the fatigued limb) hemisphere; changes were correlated with the evoked force. Also, the *gamma* band (30-50 Hz) power decrease during the SC did not return to baseline after 2 min of rest in CRF, an effect correlated with BFI score. In conclusion, altered brain electrical activity during a fatigue task in patients is associated with central fatigue during SC or fatigue symptoms, suggesting its potential contribution to CRF during motor performance. This information should guide the development and use of rehabilitative interventions that target the central nervous system to maximize function recovery.

1. Introduction

Cancer-related fatigue (CRF) is defined as "a distressing, persistent, subjective sense of physical, emotional, and/or cognitive tiredness or exhaustion related to cancer and/or cancer treatment that is not proportional to recent activity and interferes with usual functioning" [1]. CRF is the most frequently reported (30%-90%) and undertreated symptom while having the greatest adverse influence on quality of life (both during and following treatment) of all cancer symptoms [1–4].

Fatigue in cancer patients is multifactorial and may be influenced by several demographic, medical, psychosocial, behavioral, and biological factors [3, 5]. However, fatigue can still persist after ruling out comorbid, environmental, or social contributing factors, pointing to an intrinsic biological mechanism of CRF [5]. The biological etiology of CRF is not fully understood and is still the subject of active research.

Cancer and its treatment have been associated with abnormal immune and inflammatory responses and hypothalamic-pituitary-adrenal axis dysregulation causing neuroendocrine alteration and metabolic and mitochondrial cellular impairment [2, 4, 5]. Ultimately, they are hypothesized to affect the central and peripheral nervous systems with alterations in neural processes and regulations, generating fatigue and other behavioral changes [4, 5].

No unifying hypothesis has been convincingly developed to explain CRF etiology. Difficulty in defining and assessing CRF, symptom complexity, variability in expression and severity, and population heterogeneity presents unique challenges in advancing our knowledge of CRF. Physiological studies of motor fatigue may provide more objective information and additional insights into understanding the mechanisms of CRF. This is particularly relevant given the evidence supporting exercise as an effective approach for lessening CRF [6]. Our research in recent years has shown that CRF patients fatigue earlier than healthy controls during a prolonged submaximal muscle contraction [7, 8]. The relative contribution of peripheral fatigue at the muscle vs. central fatigue at the level of the brain or spine can be evaluated by measuring the twitch force (TF), an inverse measure of muscle reserve. TF is the amount of force generated by supramaximal stimulation of muscle or associated nerve at rest or during a sustained contraction; the lower the TF, the more fatigued the muscle. Interestingly, the TF and other myoelectric measures of muscle function indicated that the muscle at the end of the task was less fatigued in CRF than healthy controls, suggesting a greater amount of central fatigue [7–10]. Central fatigue in motor performance, defined as loss of voluntary activation of muscle, is complex and multifactorial [11–13]. It may arise at spinal and/or supraspinal levels from an increased inhibitory input, decreased motoneuron firing and excitability, or suboptimal cortical drive [11, 14]. One or more of these factors could be examined to better understand CRF.

Neuroimaging studies in healthy populations can provide insight into the supraspinal modulation of motor fatigue [15–19]. Neuroimaging findings have also helped monitor brain activities under fatigue conditions in clinical populations such as chronic fatigue syndrome [20, 21] and multiple sclerosis [22–25]. Similarly, functional [26–28] and structural [29] brain changes have been correlated with fatigue symptoms in cancer suggesting a potential cortical origin. However, no CRF study has examined brain signals correlating with fatigue during a physical fatigue task. To further support and understand the neural mechanisms of the central origin of CRF, we analyzed and compared EEG data collected between cancer patients and healthy controls during a motor fatigue task activity in a prior research project [7]. A greater understanding of the neural plastic changes occurring during the disease process will help guide the development and use of effective rehabilitative interventions to improve CRF.

2. Materials and Methods

2.1. Experimental Protocol. After participants completed the BFI, the maximum elbow flexion force (MEF) and handgrip strength were measured from the right dominant arm/hand. Participants were then asked to do the sustained contraction (SC) fatigue task by maintaining an isometric elbow flexion at 30% MEF until exhaustion (defined as a failure to maintain 30% MEF for >5 seconds). The M-wave, i.e., maximum electric stimulation-evoked compound muscle action potential, from the brachioradialis, one of the elbow flexors, was recorded before and after the SC by superficially stimulating the radial nerve on the lateral side of the upper arm. The evoked twitch force (TF) was acquired at rest (TF_{pre}) before the SC, at 30-second intervals during the SC, and at rest after the SC (TF_{post}) by stimulating the biceps brachii muscle with maximal intensity. The post to pre twitch ratio ($TF_{ratio} = TF_{post}/TF_{pre}$) was then computed; lower value < 1.0 indicates more muscle fatigue, i.e., peripheral fatigue at time of exhaustion. High-density EEG was also continuously recorded before, during, and after the SC. The MEF force was measured again immediately after the SC. Detailed procedures and results for all measures except brain activity (EEG) can be found in [7].

2.2. EEG Measurements. Brain signals were recorded using a high-density 128 channel EEG data acquisition system from Electrical Geodesics, Inc. (Eugene, OR, USA) with impedance kept below 10 Kohms for motor regions. EEG signals were amplified (×75,000), band-pass filtered (0.1-100 Hz), and digitized (500 sample/s) using Neuroscan (Compumedic NeuroScan, Charlotte, NC). As illustrated in Figure 1, the EEG data were processed as follows. The data were band-pass filtered (1–50 Hz), and average referenced after noisy or high impedance channels were removed. Data were visually inspected to remove eye blinks, muscle, and movement artifacts. In particular, the experimental protocol involved electric stimulation-evoked twitch force of the biceps brachii, a major elbow flexor muscle about every 30 s during the SC to investigate the central vs. peripheral progression of fatigue (see [7] for details). These stimulations created large transient electrical and muscle artifacts typically lasting a few seconds. Typically, 10 to 30% of the data were removed throughout the fatigue task. Postprocessing and data analysis were then performed (1) at baseline rest, i.e., before the SC (BL), (2) during the SC, and (3) during the recovery period just after the SC (RC1) and 2 min later (RC2). The duration of the SC was further divided into 3 equal segments (based on signal duration before artifacts rejection) corresponding to the beginning, middle, and end of the SC (SC1, SC2, and SC3) to study fatigue progression [7].

This processed data was then used to extract the source activity waveforms of two equivalent brain dipoles representing activities in the left (LH) and right hemisphere (RH) brain regions associated with contralateral and ipsilateral sensorimotor cortices, by applying the FOCUS method and toolbox [30, 31] from the Brain Electrical Source Analysis (BESA, version 5.1 MEGIS) software [32]. The FOCUS method essentially removes the volume conduction effect on the recorded data by modelling brain source contributions to scalp activities using spatial deconvolution. Source localization was modelled as two independently time varying and

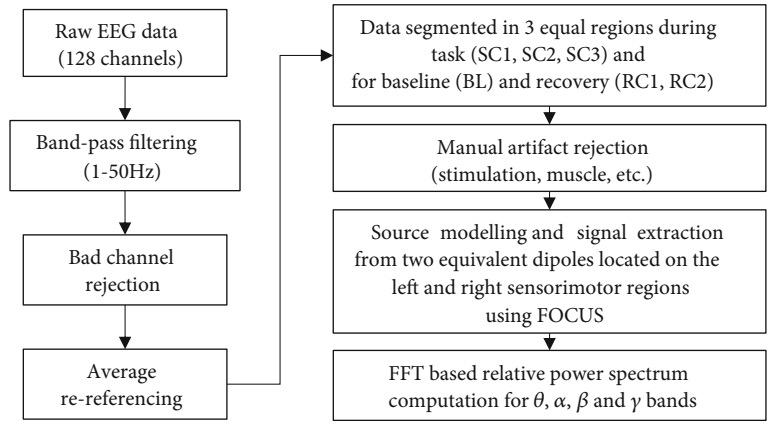

Figure 1: EEG processing pipeline.

spatially stationary equivalent dipoles with directions perpendicular to the scalp surface under electrodes C3 and C4, using a simplified spherical head model. Frequency power (nAm^2) of the LH and RH source activity was then computed for *theta* (4-8 Hz), *alpha* (8-12 Hz), *beta* (12-30 Hz), and *gamma* (30-50 Hz) bands using Fast Fourier Transform (FFT). Given the high within and between subject variability in total power, results in each band are presented as relative power to the total power in each time segment for each participant.

2.3. Statistical Analysis. Between-group comparisons of BFI and participants' demographics were performed using simple *t*-test for continuous and Pearson's Chi-square for categorical data. Linear mixed effect regression models were used to model EEG data for each frequency band and each hemisphere. To confirm model validity, residuals were plotted to verify distribution normality. We used *F*-tests (type 3 tests) to evaluate the effect of group, time, and their interaction (denoted by "Group," "Time," and "Time*Group," respectively). Whenever significance was observed, post hoc contrast analyses were done for (1) within group (time effect with respect to baseline), (2) between-group comparisons for each time point, and (3) group by time interaction. The latter was done from baseline (BL) to end of contraction or recovery (SC3, RC1, and RC2) or from beginning to end of the SC (SC3 vs. SC1) to study the effect of and recovery from fatigue during contraction. Finally, whenever this post hoc analysis revealed a significant group by time interaction, we investigated the relation between EEG frequency power changes and measure of perceived fatigue (BFI scores), endurance (SC duration), and central vs. peripheral fatigue (twitch force ratio) by performing a Pearson's correlation analysis. To ensure that the correlation was not artificially driven by large group differences, we checked that the correlation remained with CRF only. To avoid spurious results (due to lack of normality or presence of outlier), results were checked against nonparametric Spearman's correlation and recomputed without outliers if necessary. Given the exploratory nature of the study, no adjustment for multiple comparisons was made.

3. Results

3.1. Participants. Sixteen patients with stage 3 or 4 solid cancers (lung, breast, and/or gastrointestinal cancer) referred to palliative medicine, and 16 age- and gender-matched healthy volunteers were recruited to the study. Eligible cancer patients had not had chemotherapy, radiation therapy, or surgery in the preceding 4 weeks. Detailed clinical and demographic information about the study participants is available elsewhere [7].

Most cancer patients had significant cancer-related fatigue (CRF) determined by the brief fatigue inventory (BFI) [33]. Thirteen of the 16 patients had moderate to severe levels of fatigue with scores ≥ 4. In comparison, all healthy controls had mild levels of fatigue with score < 4. Inclusion criteria was as follows: fatigue not caused by another factor such as anemia or depression, hemoglobin levels ≥ 10 g/dL, not on psychostimulants or antidepressants, not depressed based on the medical record and self-report assessment to the validated screening question "are you depressed?" [34], cancer – related weight loss by history $< 10\%$, no cognitive impairment, severe polyneuropathy, amyotrophy, myasthenic syndrome determined clinically, or significant pulmonary compromise as defined by oxygen dependence. The study was approved by Cleveland Clinic's Institutional Review Board, where the study was performed.

3.2. Population Demographics and Fatigue Profile. There was no statistical difference between CRF and the control groups in age (mean ± standard deviation of 61.7 ± 10.3 yrs for CRF vs. 55.3 ± 10.9 yrs for controls, $p = 0.10$), gender proportion (9/16 vs. 11/16 female patients, Pearson's Chi-square $\chi^2(1.32) = 0.533$, $p = 0.46$), and BMI (25.7 ± 5.3 kg/m^2 vs. 28.8 ± 6.5 kg/m^2, $p = 0.16$). Patients reported feeling more fatigued than controls at the time of measurement (BFI score of 5.0 ± 1.8 vs. 0.9 ± 1.0, $p \leq 0.001$).

3.3. EEG Outcome. As previously reported, CRF participants felt fatigue and ended SC task earlier than controls [7]. Therefore, the total duration of the fatigue task and consequently each resulting EEG data segment SC1, SC2, and SC3 (before artifact rejection) were shorter in CRF and varied

Table 1: Linear mixed model type 3 F-tests to evaluate the effect of time, group, and their interaction for all four frequency bands and both left and right hemispheres.

		Theta		*Alpha*		*Beta*		*Gamma*	
		Left	Right	Left	Right	Left	Right	Left	Right
Time	$F(5.150)$	13.1	6.71	1.41	1.24	2.39	1.91	16.11	17.56
	p	**<0.001**	**<0.001**	0.22	0.29	**0.04**	0.1	**<0.001**	**<0.001**
Group	$F(1.30)$	4.58	3.62	0.19	0.96	1.52	0.54	0.21	0.001
	p	**0.04**	0.07	0.67	0.33	0.23	0.47	0.65	0.97
Group*time	$F(5.150)$	2.61	4.15	3.36	0.63	3.31	1.39	2.74	1.98
	p	**0.03**	**0.001**	**0.007**	0.68	**0.007**	0.23	**0.02**	0.08

between participants (fatigue task duration was 313 ± 145 s for CRF vs. 529 ± 131 s for controls, $p < 0.001$).

Table 1 shows that the relative power analyzed using the linear mixed model changed significantly over time for both left (LH) and right (RH) hemispheres for the *theta* (4-8 Hz) and *gamma* (30-50 Hz) bands and the LH *beta* (12-30 Hz) band, but not for the *alpha* (8-12 Hz) band. Furthermore, group difference was significant for the LH and trended toward significance ($p = 0.07$) for RH *theta* band only. The group by time interaction showed significant effects for the LH (contralateral to the performing arm) for all 4 frequency bands and the *theta* band only for RH, with some trend toward significance ($p = 0.08$) for the RH *gamma* band.

The results of post hoc analyses using contrasts are demonstrated in Figure 2. In the within-group analysis, a *theta* band power (Figures 2(a) and 2(b)) increase was seen during the entire contraction (SC) for both hemispheres in both groups, but to a greater extent in controls, before returning to baseline during recovery. The between-group difference shows that the *theta* EEG band power was significantly greater in controls than CRF at the beginning of the SC (SC1) for the LH and end of the SC (SC3) for both hemispheres.

In contrast, no significant effect was observed for the *alpha* band (Figures 2(c) and 2(d)) except during recovery for the LH (RC1) where CRF showed a significant marginal increase in power ($p = 0.04$) and a significant group difference ($p = 0.05$).

The relative *beta* band (Figures 2(e) and 2(f)) power significantly increased for CRF in the RH during the SC for all three contraction periods (but marginally so for the LH, i.e., at SC2 only) before returning to baseline afterwards. In contrast, controls saw a marginal power decrease in the LH which only became significant at the end of the SC (SC3, $p = 0.02$) before returning to baseline (while no significant changes occurred in the RH). Between-group comparisons revealed that the EEG power at *beta* band differed significantly at all three SC periods for the left hemisphere and only at SC3 for the right hemisphere.

The within-group analysis also revealed that the *gamma* band (Figures 2(g) and 2(h)) power substantially decreased for both groups during the entire SC ($p < 0.01$) for both LH and RH, but did not return to prefatigue values in the CRF group after a 2min recovery period (RC2, $p < 0.003$). The between-group comparison shows that the relative *gamma* power was significantly greater at baseline in the CRF group than controls ($p = 0.01$).

The group by time post hoc analysis during the SC task (SC3 vs. SC1) reveals a trend toward significance in RH *theta* power ($t(150) = -1.821$; $p = 0.07$), where it increased in controls, but decreased in CRF.

3.4. Correlation Analyses. The group difference in LH *gamma* power at baseline (Figure 3) was not associated with any significant correlation with BFI.

Group by time effect from baseline to the end of SC (SC3) shows a significant interaction for RH *theta* ($p = 0.01$) and *beta* power ($p = 0.005$) and a trend toward significance for LH *beta* power. Subsequent correlation analysis revealed a significant positive relation between this LH and RH *beta* power change and twitch force ratio (Figures 3(a) and 3(b); $R^2 = 0.19$, $p = 0.01$ and $R^2 = 0.33$, $p = 0.001$, respectively, for the whole sample; Spearman $\rho^2 = 0.25$ and $p = 0.004$ for the RH *beta* power when including the CRF outlier; $R^2 = 0.27$, $p = 0.04$ and $R^2 = 0.22$, $p = 0.08$ for CRF only). No correlation was found with BFI or task duration.

A significant group by time effect for postcontraction compared to baseline was also found for LH *alpha* ($p = 0.006$ for RC1 and $p = 0.03$ for RC2) and LH and RH *gamma* ($p = 0.002$ and 0.006 for RC1 and 0.005 and 0.029 for RC2, respectively). Subsequent correlation analysis showed a significant negative correlation between BFI and LH, but not RH *gamma* power change (Figure 3(c); $R^2 = 0.32$ and $p = 0.001$ for the whole sample except for one CRF outlier and $R^2 = 0.21$ and $p = 0.08$ for CRF only; Spearman $\rho^2 = 0.33$ and $p = 0.001$ for the whole sample).

Finally, a significant group by time interaction was found for RH *theta* power change during the SC (SC1 to SC3) ($t(150) = -1.821$; $p = 0.07$), where it increased in controls, but decreased in CRF. This change significantly correlated with twitch force ratio (Figure 3(d); $R^2 = 0.33$ and $p = 0.001$ for the whole sample excluding one control outlier and $R^2 = 0.36$ and $p = 0.02$ for CRF; Spearman $\rho^2 = 0.48$ and $p < 0.001$ for the whole sample), but not with BFI and task duration.

4. Discussion

Our prior research found that the inability of CRF to sustain a submaximal muscle contraction as long as healthy controls is largely the result of greater central fatigue at the spinal and/or cortical levels rather than at peripheral or muscle level

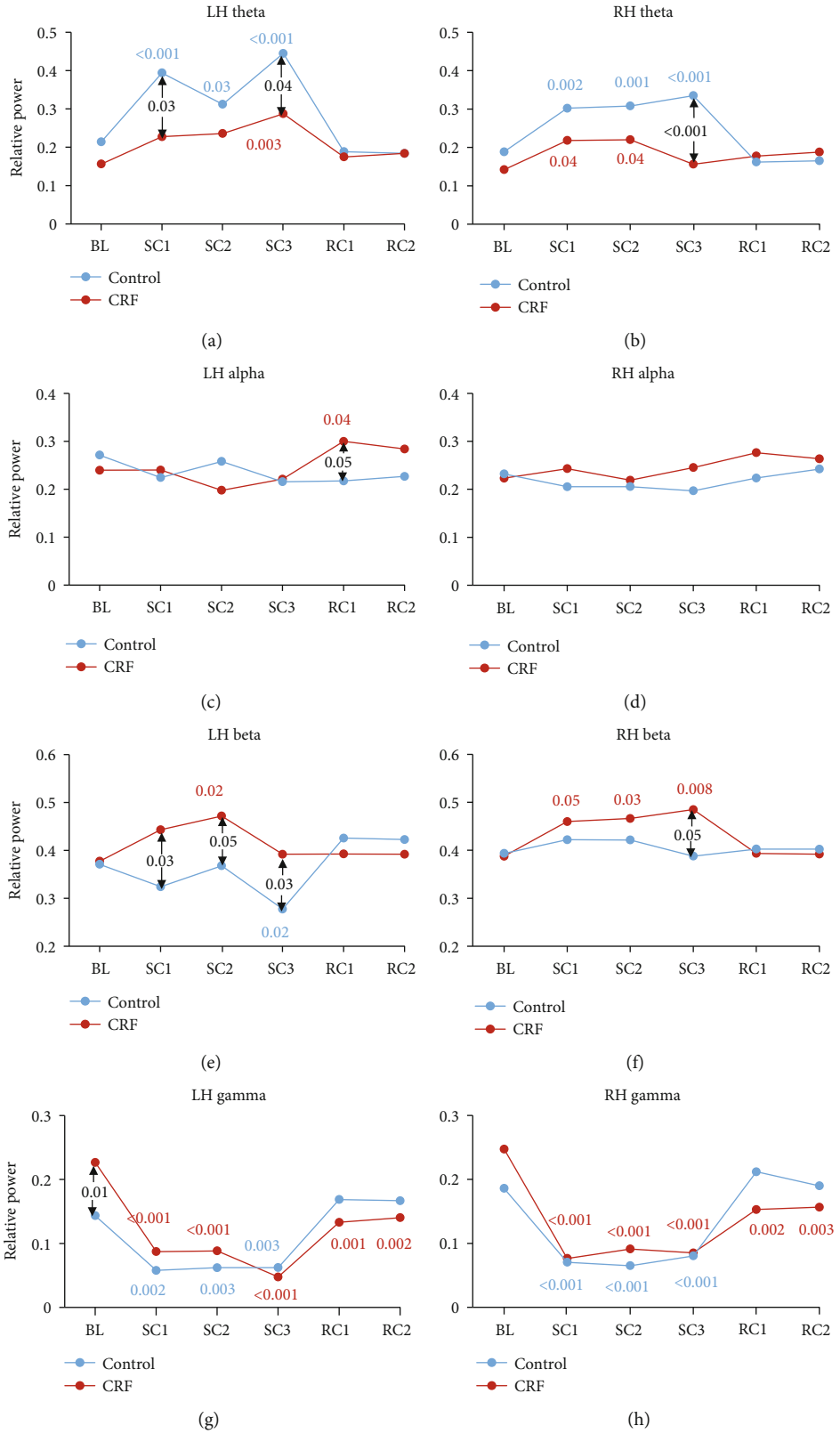

Figure 2: Relative EEG power change at each frequency band during the fatigue task for CRF (red) and healthy controls (blue) for the left (LH) and right (RH) hemispheres. Estimated marginal means are derived from the mixed linear model. Whenever significant, p values are shown in red for CRF and blue for controls for the within-group contrast analysis (compared to baseline) and in black for between-group contrast analysis. CRF: cancer-related fatigue; BL: pretask baseline; SC1, 2, and 3: beginning, middle, and end of the sustained contraction fatigue task, respectively; RC1 and 2: recovery period right after the end of the task and 2 min later.

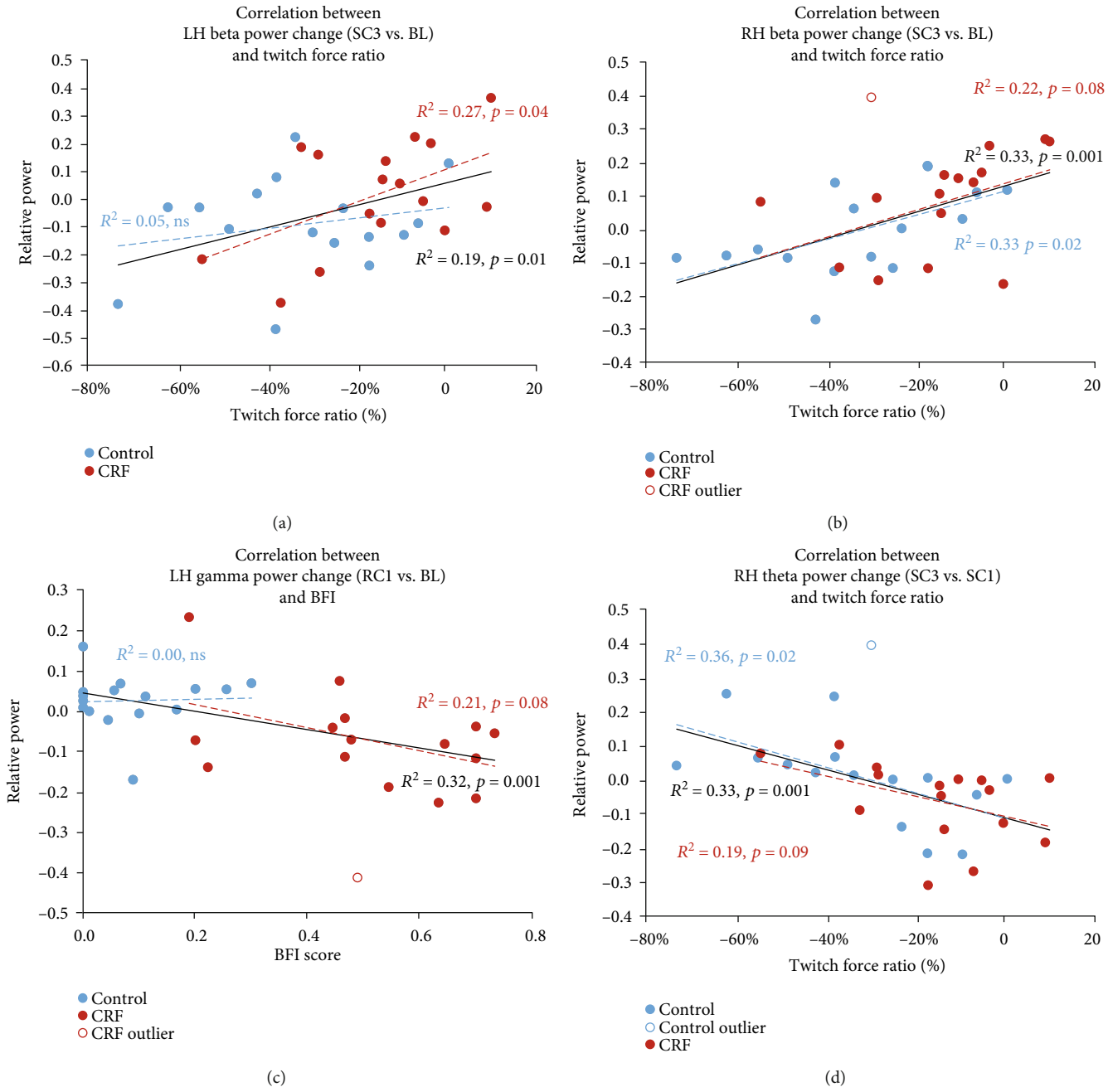

Figure 3: Linear correlation between (1) relative power change during the sustained contraction task (SC3 vs. SC1), at end of the task (SC3), or posttask (RC1) compared to baseline (BL) for CRF (red) or controls (blue); (2) twitch force ratio or BFI score. Linear fits are shown in black for the whole sample, blue for controls, and red for CRF. Outliers are shown in open circles; linear fitting was done without the outliers. CRF: cancer-related fatigue; BFI: brief fatigue inventory.

[7–10]. Here, we analyzed EEG data collected during the motor activity to assess whether it would support the central origin of CRF and improve our understanding of the supraspinal origin of the symptoms.

The main findings of the current analysis are as follows: (i) the relative LH EEG *gamma* frequency (30-50 Hz) power at baseline is significantly greater for CRF than controls; (ii) the relative LH (and somewhat RH) *theta* (4-8 Hz) power is lower, and the *beta* (12-30 Hz) power is greater in CRF than controls during the sustained contraction (SC), (iii) and this elevated *beta* power correlates with the twitch force ratio, a measure of central fatigue at the end of the SC; (iv) in contrast to healthy controls, the relative LH and RH *gamma* and LH *alpha* (8-12 Hz) power did not fully recover back to baseline in CRF after the SC; (v) this lack of recovery for LH *gamma* power in CRF was correlated with the level of overall perceived fatigue (BFI).

A greater relative *gamma* band power at baseline may point to a potential marker of fatigue at rest in CRF. This is difficult to explain, however, as it did not correlate to BFI

scores. Fatigue has been more commonly associated with *theta*, *alpha*, or *beta* bands in other pathologies, like chronic fatigue syndrome [20, 21, 35], multiple sclerosis [24, 36], and burnout patients for whom their *alpha* and/or *beta* frequency power correlated with subjective fatigue [37]. Perhaps the *gamma* band power only reflects a subjective feeling of fatigue, but does not relate to its severity or may relate to other cancer-related symptomologies.

During the SC, we observed a decrease in relative *gamma* band power for both CRF and controls. This is noteworthy as usually tonic muscle contractions are promoted by *beta* oscillations whereas changes in EEG *gamma* power occurs mainly during dynamic motor activities [38, 39], although alterations of the *gamma* band power have previously been observed during a short-duration SC [40]. It is unclear why *gamma* band power *decreased* as *gamma* wave *synchronization* (i.e., increase in its power) usually occurs during dynamic motor control task where it is thought to support visuomotor integration [38, 40, 41]. This would need further investigation.

More importantly, CRF shows abnormal lower *gamma* power in the LH and RH post-SC, as it failed to fully recover back to baseline (Figures 2(g) and 2(h)), suggesting that the altered brain signal by the fatiguing SC lingered longer in CRF than healthy controls. Furthermore, the amplitude of lack of recovery in the LH *gamma* power was negatively correlated with BFI in CRF. In other words, the more subjective fatigue, the less neural recovery from the motor fatigue indicated by the *gamma* band signal. Interestingly, the larger the abnormally elevated *gamma* band power at rest, the greater the lack of recovery ($R^2 = 0.6$ and $p < 0.001$) for CRF, suggesting a close relation between persistent symptoms of fatigue, elevated EEG *gamma* power, and subsequent lack of recovery of the signal from motor fatigue induced by the SC. Further research is needed to fully understand the underlying physiological mechanisms at play.

Controls had a greater increase in relative *theta* band activity than CRF during the SC. This was present in the LH (Figure 2(a), SC1 and SC3) for the duration of the SC, but also in the RH (Figure 2(b), SC3) at the end of the SC, i.e., at the highest level of physical fatigue. Interestingly, the increase in RH *theta* power at the end compared to the beginning of the SC seems to be inversely correlated with the twitch force ratio (Figure 3(d)). This would suggest that the RH (and to some extent LH) *theta* power may be directly correlated to the level of muscle fatigue and therefore the relative level of peripheral vs. central fatigue, which was less in CRF [7–10]. It has been previously proposed that low-frequency neural signal synchronization spanning across *delta* and *theta* bands (<8 Hz) may entrain and coordinate distant brain regions to enhance information processing when task demands increase, as well as integrate motor commands and incoming visual-somatosensory information to improve motor performance [41–43]. Greater *theta* activity may thus be consistent with neuroimaging findings of greater cortical demands on a wider motor network during fatigue to maintain task performance and force output [15, 16, 44]. This increased cortical activity may represent either (1) an increase in nociceptive groups III and IV afferent input to sensorimotor cortex from muscles as they fatigue; (2) a greater cortical drive primarily from higher-order motor cortices, such as the prefrontal and premotor areas, to compensate for the loss of drive from the primary motor cortex which must increase motor unit recruitment and firing to maintain force production as the muscle fatigues; or (3) both [11, 14, 17–19]. In this framework, the lower *theta* activity increase in CRF may be a direct reflection of reduced afferent input and thus somatosensory processing due to lesser peripheral fatigue [7, 8] or may point to a central origin of fatigue as the inability to engage and coordinate distant brain regions to respond to the increase in task demand.

This proposed explanation of response failure to increased cortical demands or decreased afferent inputs associated with lower peripheral fatigue in CRF is also consistent with the observed low *beta* power in the LH (and some RH) and the absence of LH *beta* desynchronization seen in healthy controls. *Beta* desynchronization (power decrease) is believed to reflect the release of background cortical inhibition involved in gating motor commands and somatosensory inputs during movement execution [42, 45]. Greater *beta* desynchronization has been observed in submaximal sustained fatigue compared to a nonfatigue contraction task (40% vs. 5% of MVC) and is thought to reflect increased corticospinal output to maintain consistent force and increased afferent feedback [46]. This aligns with our observed linear relationship between *beta* power desynchronization at the end of the SC and twitch force ratio. The greater the peripheral fatigue (the lower the twitch force ratio), the greater the decrease in *beta* power. Depressed *beta* power during the SC in CRF may thus reflect (1) the inability to release background cortical inhibition (or increase its excitability) to promote motor output or (2) a reduction in afferent input and thus somatosensory processing from lower peripheral fatigue/higher central fatigue associated with earlier task failure.

Electrophysiology and neuroimaging fatigue studies have been performed in other populations. However, few have been conducted during a physical task making it difficult to compare our results to those in the literature. Elevated cortical resting state activity or reduced connectivity or efficiency in the low frequency bands (*theta* in particular) has consistently been reported in chronic fatigue syndrome (CFS) [35, 47–49], especially in the sensorimotor and frontal cortical regions. Similarly, more relative *theta* activity was reported during a fatiguing contraction task in CFS [21]. Like CFS, consistent with structural and functional connectivity loss from demyelination or other neural damage, fatigue in multiple sclerosis (MS) correlates with functional connectivity impairment especially in the frontal (such as SMA) and parietal (somatosensory cortex) cortical network, suggesting a critical role of sensory processing in modulating fatigue perception [24, 50]. This impaired connectivity might explain an increase in *beta* and *theta* bands and decrease in the *alpha* band resting state EEG activity—and reduced *alpha* band coherence—in the same regions compared to control [51, 52], even though their correlation with fatigue was not explored. This connectivity loss may explain a compensatory increase in cortical activity during simple motor tasks, not only in motor areas, but also in those involved in

sensorimotor integration and attention, like prefrontal and parietal areas [36, 53–56]. In particular, Leocani et al. observed greater frontal *beta* desynchronization during movement preparation and reduced postmovement frontal and contralateral central *beta* synchronization (reflecting impaired disinhibition) in fatigued compared to nonfatigued MS patients and healthy subjects [36]; effects correlated with fatigue severity. This is consistent with the need for a greater compensatory central drive during a submaximal fatigue task [57]. This can result in a blunted response when task demands exceed compensation capacity like during a maximal fatigue task or more advanced stage of the disease [58, 59]. This overall greater activity seen both in MS and CFS during contraction (or rest) is the opposite of what we observed in CRF. This may suggest a different cortical response in CRF to a common underlying functional connectivity disruption or inefficiency. CRF may have limited cortical voluntary drive at the onset leading to greater central fatigue and early SC failure. This is consistent with some of our prior findings on the same experimental data [10, 60]. First the corticomuscular coupling, a measure of corticomuscular drive, was found to be already weaker in the first half of the task, i.e., before experiencing strong fatigue [60]. Second, there was no intrinsic pre- to postfatigue change in muscle property, including muscle electrical signals and force generation capability [10], suggesting that fatigue resulted from an inability of the central nervous system to fully activate the elbow flexor muscles in individuals with CRF. In contrast, CFS or MS patients need to exert greater cortical effort—as a compensatory mechanism—to maintain motor output (compared to healthy participants), consistent with greater perception of effort during a fatigue task in CFS [61] and MS [57, 59].

The analysis of our EEG data was limited to the sensorimotor regions around the central sulcus. Future efforts should look at neural correlates of CRF by studying brain activity and connectivity within the wide motor network regions, in particular the sensory and frontal cortical regions given their potential roles in supraspinal fatigue. To this end, advanced processing techniques, like independent component analysis or source imaging, have been successfully used to study fatigue in endurance cycling [62] and air traffic control [63]. In addition, corticomuscular coherence can study changes in brain to muscle drive or muscle to brain afferent inputs in CRF compared to healthy individuals [60]. Similarly, transcranial magnetic stimulation (TMS) on the spine and brain would also help investigate changes in brain excitability and the relative supraspinal and spinal contribution to central fatigue. These advanced and complementary processing techniques and modalities would also provide valuable information to help elucidate whether neural changes observed in CRF are a manifestation of less peripheral fatigue or a direct contributor to greater central fatigue.

Other limitations to the study include small sample size and lack of control for medications such as antibiotics, anticholinergics, corticosteroids, and opioids that may influence neuromuscular conduction. We did not screen for antibodies to acetylcholine receptors or voltage-gated calcium channels which would impair neuromuscular junction conduction.

5. Conclusion

Compared to healthy controls, CRF is accompanied by abnormal brain activities and modulations before and during a sustained fatiguing muscle contraction, which fail to fully recover right after the task. Some of those abnormal brain changes correlate with measure of subjective (BFI) and objective (twitch force ratio) fatigue. The specific neural changes in CRF would suggest that early fatigue and task failure may reflect an inability of the brain to respond to the increase in task demand or may be the result of decreased afferent inputs associated with lower peripheral fatigue in CRF. These results support our prior findings of central origin of CRF in motor performance, but also suggest supraspinal contribution to the symptoms, rather than at levels of the spinal cord motor nuclei or at the neuromuscular junction.

This information is critical to understand and help the development of effective rehabilitation. In particular, this would explain why relaxation as well as mild to moderate intensity exercise interventions including yoga, which have neuroprotective or promote neurogenesis and neuroplasticity, [64] have been found most effective at improving CRF [65–67]. Noninvasive brain stimulation such as transcranial direct current stimulation (tDCS) has shown to improve fatigue in neurological disease such as multiple sclerosis [68–70]. Its effectiveness should be similarly studied in CRF. Additional research is needed to confirm the supraspinal origin, as well as the specific brain regions or network connections most affected by CRF. This would guide the development of more effective rehabilitative interventions by helping to target specific brain regions or network to maximize functional recovery in CRF.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Didier Allexandre and Dilara Sevidova-Khoshknabi contributed equally as co-first authors.

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Review Article

Cytokine-, Neurotrophin-, and Motor Rehabilitation-Induced Plasticity in Parkinson's Disease

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Neuroinflammation and cytokine-dependent neurotoxicity appear to be major contributors to the neuropathology in Parkinson's disease (PD). While pharmacological advancements have been a mainstay in the treatment of PD for decades, it is becoming increasingly clear that nonpharmacological approaches including traditional and nontraditional forms of exercise and physical rehabilitation can be critical adjunctive or even primary treatment avenues. Here, we provide an overview of preclinical and clinical research detailing the biological role of proinflammatory molecules in PD and how motor rehabilitation can be used to therapeutically modulate neuroinflammation, restore neural plasticity, and improve motor function in PD.

1. Introduction

PD is the second most common neurodegenerative disorder generally affecting the population over 65. In fact, only 4% of cases occur before the age of 50 [1]. The disease is induced by the loss of nigrostriatal dopaminergic neurons, intracellular α-synuclein accumulation, and onset of motor symptoms such as abnormal voluntary movements, tremor, rigidity, slowness of movement, postural instability [2], and nonmotor impairments including cognitive decline [3], depression, and sleep disturbances [4].

However, recently, postmortem brain imaging and fluid biomarker investigations identified neuroinflammation as a crucial pathogenesis factor of PD [5–7]. Neuroinflammation is marked by activated microglia and reactive astrocytes within brain parenchyma and by the release of various inflammatory mediators including cytokines, chemokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS) [8]. These mediators can be secreted by microglia in the central nervous system (CNS), peripheral immune cells, and other cell types such as dysfunctional adipocytes [9, 10], sustaining the inflammatory reaction and maintaining a self-reverberating cycle. For a long time, the blood-brain barrier (BBB) was thought to be unaffected by neurodegenerative and neurological pathologies while nowadays, a growing body of evidence suggests that the BBB is pathologically modulated, allowing the penetration of peripheral macrophages, leukocytes, and systemic proinflammatory mediators, such as monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-8 (IL-8), and interferon-γ (IFN-γ) [11–15]. The overproduction of proinflammatory mediators also reduces the production of brain plasticity-related molecules, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), and the ability of CNS to adapt in response to a variety of external stimuli [16, 17]. In addition, in recent years, researchers have focused their attention on the beneficial effect of physical exercise on PD patients suggesting that exercise, through targeted training, can increase neuroplasticity and, in turn, improve patients' motor and cognitive performance [18]. Here, we intend to explore (1) the role of proinflammatory cytokines

and (2) the impact of traditional and not traditional forms of physical exercise on neuroinflammation and neuroplasticity in parkinsonian subjects undergoing motor rehabilitation.

To reach the aim of this study, publication search for literature review was conducted using the NCBI PubMed database based on the following groups of keywords: (1) Parkinson's disease, pro-inflammatory cytokines; (2) Parkinson's disease, IL-6, IL-1β, IL-8, MCP-1, and TNF-α; (3) Parkinson's disease, neuroinflammation, neuroplasticity; (4) Parkinson's disease, physical activity, neurorestoration, neuroplasticity; (5) Parkinson's disease, exercise, neurotrophic factors; (6) Parkinson's disease, exercise, BDNF; (7) Parkinson's disease, exercise, GDNF; (8) Pro-inflammatory cytokines, exercise, PD patients; and (9) Not traditional physical exercises, inflammatory state, PD patients. To be eligible for inclusion in the review, studies must have been published between 1990 and 2020.

2. Proinflammatory Cytokines in PD

Brain cytokine activity depends on several conditions such as the cellular sources and the pathophysiological context all contributing to the effects exerted on the brain. In fact, cytokines can promote apoptosis of neurons, oligodendrocytes, and astrocytes; cause damage to myelinated axons; but even initiate neuroprotective effects, independently of their immunoregulatory properties [19]. Although to date there is no evidence to support a specific role for any particular cytokine as a direct cause of neurodegenerative conditions, cytokine-driven neuroinflammation and neurotoxicity have been shown to modify the disease progression.

Among cytokines, interleukin-6 (IL-6), IL-1β, IL-8, MCP-1, and TNF-α have been the most studied in PD.

2.1. IL-1β. IL-1β is a proinflammatory cytokine produced mainly by macrophages and monocytes [20] and also by epithelial cells [21] and endothelial cells [22], and it has a key role in regulating inflammatory response to microbial stimuli such as the lipopolysaccharide (LPS) and sterile insults (e.g., hypoxia, hyperosmolarity, thermal damage, and gamma radiation) [23, 24].

It has been demonstrated that IL-1β, a part of the IL-1 family, acts on the CNS because of the permeability of the BBB [25], and it is also secreted into the CNS by microglial cells [26–28], astrocytes [29], oligodendrocytes [30], and neurons [31, 32]. Therefore, the presence of members of the IL-1 family and in particular IL-1β and its receptor in basal conditions in the CNS could suggest a normal physiological role for IL-1β. For instance, several studies demonstrate that IL-1β stimulates astrocytes and supports neuronal survival via production of neurotrophic factors [33, 34]. However, IL-1β contributes to and/or sustains the pathological processes and results upregulated in several neurodegenerative diseases. Increased IL-1β levels have been detected in the cerebrospinal fluid (CSF) and the striatum of postmortem PD patients [35] as compared to control subjects. Moreover, studies based on adenoviral vectors reported that sustained expression of IL-1β in the substantia nigra (SN) causes irreversible and pronounced dopaminergic neuronal loss and motor symptoms [36, 37], while IL-1β increase induced by acute administration of LPS in the SN was not toxic [38, 39]. Overall, these data suggest that sustained but not acute IL-1β expression has toxic effects on the SN. In addition, loss of tyrosine hydroxylase- (TH-) positive neurons was higher in animals that received both, a stimulus of LPS in the SN and 6-OHDA injection into the striatum, compared to those receiving just an acute stimulus of LPS [39].

Nonetheless, Saura and colleagues have demonstrated that an acute infusion of a high dose (20 ng) of IL-1β in the SN of rats 5 days before the injection of 6-hydroxy dopamine (6-OHDA) in the striatal region protects dopaminergic cellular bodies from 6-OHDA, does not induce microglia activation, and prevents motor dysfunctions [40]. Therefore, although most of the evidence reveals that an inflammatory stimulus previous to a neurodegenerative treatment increased neuronal cell death [36, 37, 39], under specific circumstances, protective effects cannot be ruled out.

2.2. IL-6. IL-6 is a member of the neuropoietic cytokine family with a wide range of biological activities. It is involved in the development, differentiation, degeneration, and regeneration of neurons in the central and peripheral nervous systems and can also stimulate glial cells [41, 42]. Dysregulation of IL-6 production and signalling has also been reported in several neurodegenerative diseases, including PD [43–45]. Interestingly, IL-6-mediated neuronal degeneration in the CNS [46] and IL-6-mediated biological activities [47, 48] depend, respectively, on the activation of two different types of IL-6 pathways: the " transsignalling "and "classical signalling." Classical signalling occurs when the 80 kD subunit of the IL-6 receptor, called IL-6r, binds to the protein. The binding of IL-6 to IL-6r is followed by homodimerization of the second receptor subunit, called gp130, and by the activation of two distinct signalling pathways: (1) the Janus kinase- (JAK-) signal transducer and activator of transcription (STAT) pathway (JAK/STAT signalling pathway) and (2) the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signalling pathway [49–51]. While IL-6r is only expressed by hepatocytes, neutrophils, monocytes/macrophages, and specific lymphocyte subpopulations [52], IL-6 affects many more cell types. This is possible because of "transsignalling": IL-6r exists in a soluble form, sIL-6r, which can bind to IL-6 and develop a circulating IL-6/sIL-6r complex which can induce the dimerization of the gp130 even in cells that do not possess IL-6r. Activation of the IL-6 pathway by IL-6/sIL-6r is known as transsignalling [53, 54]. The two pathways lead to two different cellular responses [55]. The classical pathway mediates anti-inflammatory signals while the transsignalling pathway mediates proinflammatory signals (e.g., IL-6 mediates neurodegeneration [46], cancer inflammatory response in the colon [56], and inflammatory bowel disease [57]). This emphasizes the importance of distinguishing between the two pathways when prescribing drugs for the treatment of neurological or neurodegenerative diseases [58].

In regard to the expression of IL-6 in PD, there are some controversial results. Several studies observed an increase of IL-6 in the nigrostriatal region of the postmortem brain

and in CSF of PD patients [35, 43, 59, 60]. In some studies, no difference in plasmatic levels of IL-6 was reported [60, 61], while others found elevated levels in PD patients with severe depression [62]. Still, one paper reported that IL-6 was at higher plasmatic levels in patients with a rapidly progressing disease compared to patients with usual progression [62]. Interestingly, it has been shown that Levodopa, in physiological concentrations, elicits an immunomodulatory effect on cells from both PD patients and controls and caused stimulation of IL-6 production [44].

2.3. IL-8. IL-8 is a chemoattractant cytokine secreted by a variety of cells (e.g., monocytes [63], macrophages [64], endothelial cells [65], dermal fibroblasts [66], keratinocytes [67], hepatoma cells [68], synovial cells [69], and chondrocytes [70]), and it is well known as an inflammatory factor which induces a chemotactic response involving infiltration of neutrophils through the BBB [71]. Moreover, activated microglia is also a potent secretory source of IL-8 and expresses CXCR2 receptor for the chemokine providing a positive feedback mechanism for sustained amplification of inflammatory response [72]. At present, few studies have examined levels of IL-8 in the PD brain. In one study of Koziorowski and collaborators, serum levels of the chemokine were measured in individuals diagnosed with idiopathic PD and in controls. The results showed that IL-8 concentrations were doubled in the diseased brain compared with the control; this difference in levels of the chemokine was significant [73]. However, a contrary finding has recently been reported. Levels of IL-8 and cytokine TNF-α were found reduced in serum from Indian PD patients relative to controls [74]. Overall, despite the relevance of neuroinflammation in the pathophysiology of PD, data is lacking on the roles of IL-8 and other chemotactic factors in the progression of the disease.

2.4. MCP-1 (CCL2). MCP-1 (CCL2), one of the most highly and transiently expressed chemokines during inflammation, is a member of the CC subtype chemokines. MCP-1 exerts its biological functions by binding to its high-affinity receptor, CCR2, which is mainly expressed by microglia, astrocytes, and brain microvascular endothelial cells (BMECs) in the brain [75, 76].

Several studies have demonstrated that MCP-1 is constitutively present in the brain. The neuronal expression of MCP-1 is mainly found in the cerebral cortex, globus pallidus, hippocampus, lateral hypothalamus, Purkinje cells, cerebellum, astrocytes, perivascular microglia, infiltrating leukocytes, cholinergic neurons of magnocellular preoptic, and in dopaminergic neurons of the substantia nigra pars compacta [77, 78]. The low expression in discrete neuroanatomical regions with classical neurotransmitters or neuropeptides suggests that MCP-1 may act as a modulator of neuronal activity and neuroendocrine functions [79].

Additionally, MCP-1 may modulate the function of the BBB components and thus affect the integrity of BBB. In accordance with this hypothesis, the MCP-1 level has been found to positively correlate with the permeability of the BBB and progression of diseases [80, 81] while the lack of MCP-1 or CCR2 prevents neuronal death, decreases BBB permeability, and improves neuronal function in some disorders, including hemorrhage and ischemia-reperfusion injury [81, 82].

It has also been established that MCP-1 is an important mediator in several neuroinflammatory and neurodegenerative brain diseases characterized by neuronal degeneration such as PD.

MCP-1 levels in the blood are heightened in PD subjects compared to controls and correlate with PD progression [83].

Furthermore, it has been shown that MCP-1 could be implicated not only in disease progression but also in pathogenesis. The Ccl2-2518A allele is associated with lower MCP-1 production and reduced transcriptional activity following IL-1β stimulation [84], and in genetic epidemiological studies, possession of this allele is associated with a delayed onset of PD compared with patients expressing the Ccl2-2518G allele [85].

2.5. TNF-α. TNF-α is a proinflammatory cytokine well known for its role in chronic peripheral and central inflammation. TNF-α functions are mediated by two receptors: TNF-R1 (TNF-RSF1a) and TNF-R2 (TNFRSF1b). TNF-R1 is expressed in most tissues while TNF-R2 is found in limited cell types including cells of the immune system, oligodendrocytes, and certain neuron subtypes [10]. Both types of receptors are also expressed in the cortex, the subventricular zone of the lateral ventricle, and the hippocampus [86]. In homeostatic conditions, the TNF-α gene expression is low but increases dramatically in stressing conditions such as infection, trauma, and pathologies. In the CNS, TNF-α regulates a wide range of cellular processes and exhibits pleiotropic effects with positive or negative outcomes on the brain depending on concentrations and physiological or pathological state [87, 88]. Among the positive effects of TNF-α, there are increased neurogenesis and synaptic transmission [10, 89]. It has also been shown to be protective of hippocampal neurons by suppressing the accumulation of ROS and maintaining intracellular calcium levels [90]. Moreover, it modulates glutamatergic transmission, supports neural progenitor cell survival by mediating antiapoptotic signals via TNF-R2, and has a role in cognitive impairment, confirmed by investigations in TNF-α knock-out mice that showed reduced learning capabilities, than wild-type mice [91–93]. However, as reported in numerous other studies, TNF-α also has a dark face. It is notably involved in myelin damages [94], in favouring glutamate excitotoxicity [95], in the inhibition of long-term potentiation in the Cornu Ammonis area 1 (CA1) and dentate gyrus of the rat hippocampus, and in decreasing neurogenesis [96–98]. Furthermore, elevated levels of TNF have been described in many neurodegenerative situations such as in Alzheimer's disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and PD [99–104].

High levels of TNF-α are found in both CSF and postmortem brain of PD patients and in animal models of PD [104–109] which may indicate that this cytokine acts as a mediator of neuronal damage. To understand the role of

TNF-α in the neurodegenerative process, genetically modified mouse models were designed, such as knock-out mice lacking TNF-α or TNFR.

Knock-out mice for the TNF-α gene showed a decrease in dopamine content loss in the striatum after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxin and no difference in TH-positive cells in the nervous system suggesting a generally detrimental effect of TNF-α on the metabolism of dopamine [110] which is TNFR-independent [111].

However, TNF-α could also play a dual role in PD: neuroprotective during the early stages of the injury and neurotoxic during the chronic phase. In fact, Gemma and colleagues found that if TNF-α was inhibited early, i.e., within one week after administration of 6-OHDA, the inhibition could be neurotoxic; if TNF-α was inhibited late, i.e., 7 to 15 days after administration of 6-OHDA, the inhibition was neuroprotective [112].

Several *in vivo* reports [113–116] show detrimental effects of TNF-α injection or overexpression on the SN, but adverse results have also been reported, depending on the type, dosage, and administration regimen of TNF-α. Acute administration of TNF-α in the SN did not induce degenerative effect [113]. In contrast, in another study in which a much higher dose was administered, loss of dopaminergic cells in the SN at 14 days post inoculation was observed [114].

In experiments where TNF-α is expressed chronically, toxic effects of TNF-α were clearly observed. For instance, rats in which this cytokine was chronically expressed by intranigral injection of an adenoviral vector encoding TNF-α had, 14 days after adenoviral inoculation, akinesia of the forelimbs and a distinct inflammatory response in the brain [115]. The subsequent study by Chertoff and coworkers confirms the discovery discussed above; in this experiment, the chronic expression of TNF-α resulted in a progressive loss of dopaminergic (DA) neurons and their terminals in the nervous system and the recruitment of monocytes/macrophages [116].

Taken together, these results indicate that long-term expression of proinflammatory levels of TNF-α, or acute but very high expression of this cytokine, appears to be necessary to induce toxic effects on the SN while lower levels have been generating neuroprotection transient against 6-OHDA toxicity in the SN and striatum [116].

3. Physical Exercise in the Rehabilitation of Parkinsonian Subjects and Its Role in Neuroplasticity

Specific rehabilitation programs, as a support to pharmacological therapies in the treatment of parkinsonian patients, were proposed in 1956 [117]. However, in the beginning, the approaches were based only on empirical experience and there was no attempt to understand the underlying neurological mechanisms. In recent years, the benefits of exercise have been found to be linked to neuroplasticity [18]. To investigate the mechanisms by which exercise induces neuroplasticity in the mammalian brain, the loss of dopamine cells is induced by targeted injections of MPTP (mouse and non-human primate) or 6-OHDA in rats. In both models [118–123], physical exercise improves motor performance, including gait speed, step length, and balance.

Studies on the neuroprotective effects of physical exercise introduced forced or voluntary exercise before, during, or immediately after administration of the toxins (6-OHDA or MPTP) and reported improved motor functions, along with the preservation of dopaminergic neurons and the restoration of dopaminergic terminals in the striatum. These improvements have been mainly attributed to either an increased level of neurotrophic factors such as BDNF or GDNF [124–126] or exercise-induced downregulation of the dopamine transporter (DAT) [119, 123]. Other factors affect/modulate the neuroprotective effects of exercise, among which the temporal interval between the lesion and the beginning of the physical training (e.g., exercise started 1 week after toxin administration fails to protect against cell loss [127]) and the extent of toxin-induced damage.

Neurorestoration is suggested as another exercise-induced process for recovery of behavioural functions, and it does not involve neuroprotection [123]. In fact, neurorestorative effects of exercise are defined as the brain's responses to exercise after the completion of toxin-induced cell death. Studies have shown that exercise increases dopamine release, increases synaptic plasticity, and decreases dopamine clearance by reducing DAT expression [119, 128, 129]. Furthermore, it has been shown that strenuous exercise, on a treadmill, reverses the reduction of dopamine D2 receptors in the dorsal striatum, which usually occurs after injury [118]. Both the restoration of dopamine D2 receptors and the increase in dopamine release are extremely important in the advanced phase of motor learning when automaticity develops [130]. Therefore, both phenomena could contribute to the neuroplastic mechanisms involved in the improvement of exercise-induced motor behaviour and restoration of automaticity.

Physical exercise also modulates glutamatergic neurotransmission. Among the crucial aspects underlying motor impairment in individuals with PD, there is the hyperexcitability in the indirect pathway induced by dopamine depletion in the striatum in response to alterations in glutamate receptor expression and neurotransmitter release [131]. Van-Leeuwen and colleagues have shown that strenuous exercise can restore the expression of glutamate receptors, including the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are modified in many neurological disease states and are considered a viable target for drug treatment [132, 133]. In addition to the effects on glutamate receptors, exercise can also alter the storage and release of glutamate in presynaptic terminals, which may also improve circuit function and reduce the increased inhibitory drive of the dopamine-depleted striatum [134–136]. Thus, these findings suggest that exercise, through its effects on neurotransmitters and their receptors, could help restore the neurophysiological properties of synapses within the damaged striatum that are necessary for normal motor learning and motor activity [18].

In summary, exercise is generally accepted as an intervention that could help both motor and nonmotor complications of PD, but it should be emphasized that not all types of rehabilitation approaches could facilitate neuroplasticity and behaviour in individuals with PD. Indeed, experience-dependent neuroplasticity is largely dependent on the intensity, repetition, specificity, difficulty, and complexity of the practice, and it is very likely that patients with PD need more time to achieve effective learning and automation. A precedent study by Frazzitta and colleagues [137] demonstrated that the rate of recurrence of physiotherapy sessions (2 daily sessions, 5 days a week for 4 weeks) induces beneficial effects that persist for a follow-up period of 12 months, with a reduced need to increase the doses of Levodopa. This result would suggest that the frequency of rehabilitation intervention is a critical factor, which could influence the natural progression of motor impairment in PD.

The study of Tinazzi and colleagues (2019) based on a four-week trunk-specific exercise program in PD patients with pronounced forward trunk flexion has confirmed the importance of intensive and specific physiotherapy. Rehabilitative protocols lasted 4 weeks (60 min/day, 5 days/week) and have led to improved passive and active control of the trunk that was maintained at one month post treatment [138].

Similarly, Corcos and colleagues [139] reported that progressive resistance exercise improved motor subscale Unified Parkinson's Disease Rating Scale (UPDRS-III) scores in PD patients with an effect lasting up to 2 years. Therefore, it could be hypothesized that the association of periodic intensive rehabilitation courses with pharmacological treatment should be considered one of the best options for the treatment of PD patients. To date, however, there is still a need for a general consensus on which is the best treatment modality (type-frequency-intensity) and on the most significant outcome measures [140].

3.1. Effects of Exercise on Cytokines and Neurotrophin Levels. Here, we intend to focus on the effects of exercise on altered levels of proinflammatory cytokines and GDNF and BDNF.

Neurotrophins are a group of proteins having the ability to stimulate survival, cell growth, and maintenance of the functional capacities of specific neuronal populations [141]. Initially, neurotrophins are synthesized as precursor proteins (proneurotrophins) and, because of the involvement of several enzymes, are converted into their mature form and released into the extracellular space [142]. Each of these mature proteins forms a complex with a twin molecule forming a dimeric structure that allows the activation of specific receptors [143]. Neurotrophins act through two types of receptors: tyrosine kinase receptors, with high affinity for mature neurotrophins, and p75 receptors, with low affinity for mature and high affinity for immature forms. Previous studies have suggested that proneurotrophins, through the p75 receptors, exert opposite biological effects with respect to mature proteins, and therefore, the proteolytic cleavage of proneurotrophins may represent a control mechanism that orchestrates the activity of neurotrophins [144]. Furthermore, these proteins are able to self-regulate their production as well as regulate the production of other members of this group of proteins [145, 146].

The most studied neurotrophic factors in PD are GDNF and BDNF.

GDNF is a neurotrophic factor purified for the first time from a rat glioma cell line (B49) [147] and belongs together with neurturin (NRTN), artemin (ARTN), and persephin (PSPN) to the family of GDNF ligands (GFL) belonging in turn to the superfamily of transforming growth factor β (TGF-β) [148]. In recent years, both the GDNF and the GFL ligands have been investigated due to their involvement in the survival of dopaminergic and noradrenergic neurons. However, GDNF besides acting on dopaminergic neurons promotes the survival of many other neuronal populations including motor and enteric neurons, noradrenergic and serotonergic cell population, and peripheral sensory and autonomic neurons. In addition, GDNF is expressed in brain regions that receive catecholaminergic afferents [149], such as the striatum and thalamus [150, 151].

Furthermore, studies performed on rat and mouse models of PD showed the neurorestorative properties of GDNF [152, 153]. In nonhuman primate PD models, GDNF augmented the sizes of nigral DA neurons that were 20% larger, with an increased fiber density, and it improved parkinsonian symptoms such as bradykinesia, stiffness, balance, and posture [154, 155].

Furthermore, the trophic effects of GDNF have been described as TGF-β-dependent. Indeed, TGF-β acts as a modulator of GDNF signalling and participates in the translocation of GDNF family receptor-α (GFRα) coreceptors in the cell membrane. The association between ligand and coreceptor forms the GDNF-GFRα complex that can interact with the neural cell adhesion molecule (NCAM) receptors or with a transmembrane tyrosine kinase (RET (REarranged during Transfection)) dimer, inducing their homodimerization and tyrosine autophosphorylation and initiating the intracellular signalling process. Hence, a series of cascades occur, including the activation of the nonreceptor tyrosine kinase Fyn- (Fyn-) focal adhesion kinase- (FAK-) MAPK signalling pathway by the GDNF-GFRα-NCAM complex and the activation of the rat sarcoma virus GTP-binding protein- (RAS-) MAPK-phosphoinositide 3-kinase (PI3K) signalling pathway by the GDNF-GFRα-RET complex [156, 157]. These cascades play a role in the control of neurite outgrowth [158] and in neuronal growth and survival through the activation of the cAMP response element-binding protein (CREB) and the protein kinase B (PKB), involved in cell proliferation and transcription [156, 159]. Furthermore, GDNF also appears to be able to modulate microglia activation through GDNF family receptor α1 (GFRα1). Thus, GDNF triggers signalling cascades, which are responsible for inhibiting microglia activation [160].

Because of these promising effects on PD, researchers have investigated several means able to increase GDNF levels.

The direct delivery of GDNF to the brain region affected in PD seems to optimize the chances of obtaining therapeutic efficacy. Using different viral vectors and different animal models including adeno-associated viral vectors (AAV) in

rat models of PD [161], AAV in nonhuman primates [162], and lentivirus [163] and adenovirus [164] in rats, the neurorestorative effects of GDNF were carefully demonstrated. Although these findings are promising, the results from clinical trials are not very encouraging. For example, a study based on monthly intracerebroventricular injections of GDNF reported no improvement and several side effects [165]. However, another study where GDNF was administered directly into the putamen showed an improvement in motor function as well as an increase in dopamine uptake measured by positron emission tomography (PET) without any side effects [166].

So far, the clinical evaluations of GDNF treatments in patients with PD have been inconsistent, potentially due to insufficient distribution of GDNF throughout the nigrostriatal system [167–169]. In order to increase GDNF nigrostriatal distribution, we conducted a study using an implantable and removable encapsulated cell system able to deliver targeted and long-lasting *de novo* synthesized high levels of human GDNF into the striatum of 6-OHDA-lesioned rats and Goettingen miniature pig. GDNF was distributed throughout the striatum, and this massive spreading of the protein led to almost complete protection of dopaminergic neurons in the damaged SN and preservation of TH-positive fibers in the striatum. Furthermore, these same animals demonstrated a slow and steady improvement in motor performance when evaluated on 3 separate neurological tests (cylinder, placing, and stepping tests). Our data demonstrated also that a part of the motor recovery is explained by the germination or regeneration of residual dopaminergic terminals postinjury [170]. Beneficial effects were observed when the same therapeutic approach was investigated into the hippocampus of pilocarpine-treated rats [171, 172].

Thus, long-term targeted release of GDNF over the majority of the nigrostriatal system could represent an interesting and attractive option for treatment of PD.

Another valuable ally for increasing GDNF release is physical exercise [125]. A very recent study also highlighted the ability of controlled exercise on a treadmill in mice to increase the striatal content of GDNF as well as normalize striatal levels of tyrosine hydroxylase and attenuate L-DOPA-induced dyskinesia (LID [173]), thus providing the first indication that the antidyskinetic effects of exercise may lead to an increase in striatal GDNF levels [174].

The other most studied neurotrophic factor in PD is BDNF. BDNF supports the survival and the differentiation of dopaminergic neurons and protects them from neurotoxin-induced degeneration [175]. Many studies have documented some evidence of a decreased expression of BDNF in different neurodegenerative diseases [176, 177]. PD patients present lower concentrations of BDNF mRNA and protein in the substantia nigra pars compacta than healthy controls [178, 179]. On the contrary, some studies reported an increase of BDNF levels in the serum of PD patients, especially in moderate to severe stages of the disease [180, 181]. This could happen because the CNS to counteract neuronal loss would increase BDNF production resulting in enhanced serum levels of the protein. However, there is no direct evidence that supports this hypothesis. The onset and progression of PD are also associated with neuroinflammation. Sawada and coworkers have found a notable increase of microglial cells in the hippocampus, amygdala, and entorhinal cortex of PD patients, which was associated with a decrease of BDNF mRNA expression and increased IL-6 in those regions. Moreover, they have also shown increased levels of IL-1β, interleukin-2 (IL-2), IL-6, and TNF-α in the striatum of PD patients associated with decreased BDNF protein levels in the same structure [182]. However, there is no evidence on how changes in BDNF levels in the brain affect the progression of PD, and further analysis of the interaction between proinflammatory cytokines and BDNF levels is necessary.

A research field in continuous development focuses on studying the effects of exercise on BDNF level changes in healthy adult populations [183, 184] and in people affected by neurodegenerative disease [185, 186].

Exercise-induced BDNF release seems to carry out a crucial role in neuroplastic effects of rehabilitation interventions in humans with neurodegenerative disease, particularly with PD [183, 187–189], and it is believed that the physiologic mechanisms underlying exercise-induced BDNF changes in PD could include long-term potentiation (LTP) and long-term depression (LTD) mechanisms [190, 191].

In fact, it seems that BDNF plays a complicated role in both LTP and LTD and contributes in different ways to short-term and long-term plasticity: initially, the pro-BDNF binds to two postsynaptic receptors: the tyrosine kinase B (TrkB) receptor and the p75 receptor. TrkB activation facilitates the induction of LTP [192] while p75 receptor stimulation modulates the N-methyl-D-aspartate (NMDA) activity that promotes the subsequent induction of LTD [193]. Thus, although its action is particularly complex, BDNF is a major player in synaptic plasticity.

In order to explore if the neuroprotection offered by exercise is BDNF-dependent, Gerecke and colleagues (2010) studied the effectiveness of voluntary physical training with a running wheel in mice on a 90-day program. Mice were divided into two groups: mice with heterozygous deletion of the BDNF gene and wild-type mice. Only the second group showed neuroprotection against exposure to the toxin inducing dopamine cell loss [194]. Researchers also analysed voluntary training in PD mice after periods of 30, 60, or 90 days. The running training for 90 days best promoted a neuroprotective effect on dopaminergic cells showing only a 9% loss of DA neurons while loss of DA neurons was more consistent in animals that underwent 30 days or 60 days of voluntary training [194].

A different research team has demonstrated that physical exercise reduces the 6-OHDA-induced damage acting on BDNF receptors. In fact, blocking of BDNF receptors causes enhanced postlesion nigrostriatal dopaminergic cell loss, quantified as a reduction in the expression of TH [126, 191].

Finally, clinical data on the impact of physical exercise on reducing PD-related proinflammatory cytokine levels received increasing attention over recent years; in particular, investigations focus on the modulation of inflammatory markers as potential molecular mechanisms involved in the beneficial effects of exercise on PD patients.

Cadet and colleagues showed that cyclical exercise, performed for months, leads to a significant increase in the plasma level of anti-inflammatory signal molecules, such as interleukin-10 (IL-10) and adrenocorticotropin, while plasma levels of proinflammatory cytokines such as IL-1 and IL-6 were not affected. Additionally, this cyclic exercise protocol has also been shown to improve fine motor skills. These data suggest that cyclical exercise induces the formation of anti-inflammatory signalling molecules, which appear to be associated with relieving of some clinical impairments of PD [195].

Two more recent studies (years 2017 and 2018) also showed that alternative and not traditional physical exercises such as Qigong, an oriental mind-body exercise, or physical exercise in water can improve the inflammatory state of PD.

In this study by Moon and colleagues, ten subjects with PD were recruited and then randomly assigned to one of the two groups who received six weeks of Qigong intervention (experimental group) or sham Qigong (control group). After the intervention, the serum level of TNF-α in the experimental group was significantly reduced in all subjects, and there is a stabilized sleep pattern suggesting that TNF-α can potentially affect sleep quality in people with PD [196].

Pochmann and colleagues instead focused on exploring the molecular mechanisms underlying the improvement of motor symptoms and functional mobility in water-based exercise interventions in patients with PD. The authors reported higher levels of the proinflammatory cytokines IL-1β and MCP-1 in patients with Parkinson's compared to the control group and a reduction in the levels of these proinflammatory cytokines after an aquatic physiotherapy program for 1 month, two times a week (60 min/session). These data support the idea that the inflammatory state is linked to PD and that proinflammatory cytokines could be considered promising biomarkers for the diagnosis and progression of this condition [197].

4. Conclusion

In conclusion, both traditional and not traditional forms of exercise have been shown to be important for improving motor function, facilitating neuroplasticity, and reducing neuroinflammation in PD. Further investigations are needed to broaden our knowledge on the mechanisms through which specific physical training induces neuroplasticity, eventually leading to a deeper knowledge of its role in interfering with the disease progression and to identify novel therapeutic targets to finally improve the effects of pharmacological approaches of PD.

Abbreviations

AD:	Alzheimer's disease
ALS:	Amyotrophic lateral sclerosis
AMPA receptors:	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ARTN:	Artemin
AVV:	Adeno-associated viral vectors
BBB:	Blood-brain barrier
BDNF:	Brain-derived neurotrophic factor
BMECs:	Brain microvascular endothelial cells
CA1:	Cornu Ammonis area 1
CNS:	Central nervous system
CREB:	cAMP response element-binding protein
CSF:	Cerebrospinal fluid
DA neurons:	Dopaminergic neurons
DAT:	Dopamine transporter
ERK:	Extracellular signal-regulated kinase
FAK:	Focal adhesion kinase
Fyn:	Nonreceptor tyrosine kinase Fyn
GDNF:	Glial cell line-derived neurotrophic factor
GFRα:	GDNF family receptor-α
GFRα1:	GDNF family receptor α1
GFL:	GDNF family
JAK/STAT signalling pathway:	Janus kinase- (JAK-) signal transducer and activator of transcription (STAT)
IFN-γ:	Interferon-γ
IL-1β:	Interleukin-1β
IL-2:	Interleukin-2
IL-6:	Interleukin-6
IL-8:	Interleukin-8
IL-10:	Interleukin-10
LID:	L-DOPA-induced dyskinesia
LPS:	Lipopolysaccharide
LTD:	Long-term depression
LTP:	Long-term potentiation
MAPK:	Mitogen-activated protein kinase
MCP-1:	Monocyte chemotactic protein-1
MPTP:	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS:	Multiple sclerosis
NCAM receptors:	Neural cell adhesion molecule receptors
NMDA:	N-Methyl-D-aspartate receptor
NRTN:	Neurturin
6-OHDA:	6-Hydroxy dopamine
PI3K:	Phosphoinositide 3-kinase
RNS:	Reactive nitrogen species
ROS:	Reactive oxygen species
SN:	Substantia nigra
PD:	Parkinson's disease
PET:	Positron emission tomography
PKB:	Protein kinase B

PSPN:	Persephin
RAS:	Rat sarcoma virus GTP-binding protein
RET:	REarranged during Transfection
TGF-β:	Transforming growth factor β
TH:	Tyrosine hydroxylase
TNF-α:	Tumor necrosis factor-α
TrkB:	Tyrosine kinase B
UPDRS-III score:	Unified Parkinson's Disease Rating Scale score.

Conflicts 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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Research Article

Hyperexcitability of the Nucleus Accumbens Is Involved in Noise-Induced Hyperacusis

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Reduced tolerance to sound stimuli (hyperacusis) is commonly seen in tinnitus patients. Dysfunction of limbic systems, such as the nucleus accumbens (NAc), may be involved in emotional reactions to the sound stimuli in tinnitus patients. To study the functional changes in the NAc in hyperacusis, we have examined the neural activity changes of the NAc using c-Fos staining in an animal model of hyperacusis. The c-Fos staining was also examined in the medial geniculate nucleus (MGN), a central auditory pathway which has neural projections to the NAc. Postnatal rats (14 days) were exposed to loud noise (115 dB SPL, 4 hours for two consecutive days) to induce hyperacusis ($n = 4$). Rats without noise exposure were used as the controls ($n = 4$). After P35, rats in both groups were put in a behavioral training for sound detection. After they were trained to detect sound stimuli, their reaction time to noise bursts centered at 2 kHz (40-110 dB SPL) was measured. Rats in the noise group showed a significantly shorter reaction time than those in the control group to the noise bursts at high intensities, suggesting the noise exposure induced hyperacusis behavior. The c-Fos expressions in the NAc and the MGNs of the noise group were significantly higher than those of the control group. Our results suggested that early-age noise exposure caused hyperactivity in the NAc and the MGNs which may induce the loudness increase in these rats.

1. Introduction

Tinnitus is a phantom sound perception which occurs when there is no external sound in the surrounding environment. Tinnitus patients typically also experience declined sound tolerance or panics to loud sound, known as hyperacusis [1–4]. Patients who suffer from tinnitus and hyperacusis often share the limbic-associated psychological profiles with an increased tendency to anxiety, fatigue, and depression [5–9]. These anxiety disorders can exacerbate the severity of their tinnitus and hyperacusis symptoms [10].

The nucleus accumbens (NAc), a major part of the ventral striatum, is a key structure involved in mediating emotional processing. The NAc receives multiple projections from many brain areas, including the nucleus of the central auditory system, such as the medial geniculate nucleus (MGN) of the thalamus. Brain imaging studies found the structural and functional abnormalities of the NAc in tinnitus patients [11, 12]. Evidence suggested that the NAc can regulate the limbic-auditory interactions and is involved in the occurrence of tinnitus [13]. Recent studies also found that the harshness of tinnitus and hyperacusis is related to the abnormal neural excitability in NAc which causes emotional changes to sound [14–17]. However, how the functional changes in the NAc modulate sound loudness perception is not yet clear.

In our recent studies, we found that early-age hearing loss can cause hyperacusis [18]. Rats with moderate-to-severe hearing loss showed shorter reaction time compared to rats without hearing loss. Physiological studies suggested that hyperexcitability of the central auditory system may be involved in sound behavioral changes [19, 20]. To further

understand whether the functional changes of the limbic system are involved in the sound loudness changes, in this study, we used c-Fos immunostaining to detect the neural activity in the NAc and the MGNs in the rats with behavioral evidence of hyperacusis.

2. Materials and Methods

2.1. Animals and Noise Exposure. Eight neonatal male Sprague-Dawley rats (Harlan Laboratories Inc.) were used in this experiment. They were randomly divided into the control group ($n = 4$) and the noise group ($n = 4$). The care and use of animals were approved by the Institutional Animal Care and Use Committee at State University of New York at Buffalo and conformed to the guidelines issued by the National Institutes of Health.

At postnatal 16 days (P16), rats in the noise group were exposed to a narrow band noise at 115 dB SPL centered at 12 kHz (1 kHz bandwidth) for 4 hours each day in two consecutive days. The sound stimuli were generated by a sound processor (RP2, TDT, Alachua, FL, USA) and presented by a loud speaker (GMI D-49, GMI Sound Crop., NY) placed 10 cm up from the rat's head. The output of the speaker was calibrated by a sound level meter coupled to a half-inch condenser microphone (Model 824 Audiometer, Larson Davis).

2.2. Auditory Brainstem Response (ABR) Recording. ABR was used for hearing evaluation for both groups at P35. The hearing test was conducted in a sound attenuation booth, and the rats were anaesthetized with a mixture of ketamine (50 mg/kg) and xylazine (6 mg/kg). Stainless steel needle electrodes (Grass Technologies) were used for the ABR recordings. The noninverting electrode (+) was placed at the vertex, the inverting electrode (-) was inserted near the pinna of the testing ear, and the ground electrode was inserted near the pinna of the contralateral ear. The TDT System 3 (BioSigRP, Tucker-Davis Technology, Alachua, Florida, USA) was used for sound generation and data acquisition. Tone bursts (2 ms duration, 0-100 dB SPL) were used to obtain thresholds at 2, 4, 8, 16, and 32 kHz. The ABR thresholds were obtained by using a step of 5 dB SPL to identify the lowest intensity that elicited a repeatable response.

2.3. Sound Detection Training. At P35, rats in both groups were trained for sound detection test using a two-choice operant conditioning task. The detailed behavioral training method was given previously in our published paper [18, 21]. The operant conditioning training apparatus was built using modules from Med Associates Inc. (St. Albans, VT, USA) and was controlled by TDT Hardware (Tucker-Davis Technologies, Alachua, FL, USA) with custom software. The training box had a head entry (nose-poke) used for initiating sound stimuli. Two food dispensers with infrared head-entry detectors were installed on each side of the nose-poke along with a loud speaker on the ceiling of the training box (Fostex FT28D, Tokyo, Japan).

The rats were in food restriction before the behavioral training, and the training was reinforced by the palatable food pellets (Bio-Serv, NJ, USA). During the training, first, they need to initiate a sound stimulus by poking the middle head entry. Then, they need to poke the right food dispenser (H-side) upon perceiving a loud sound (90 dB SPL) and the left food dispenser (L-side) for a soft sound (50 dB SPL). Poking the correct side of the food dispenser was rewarded with food pellets; poking the wrong dispenser, no food pellets were rewarded and they could not start a new trial in 10 seconds. To prevent rats from randomly poking the nose-poke without paying attention to the acoustic stimuli, rats must keep their noses in the head entry for 1 second until a sound was presented. Withdrawing from the nose-poke less than 1 second would not trigger an acoustic stimulation and the food pellet would not be released. The loud and soft sound stimuli were presented in a random order during the training.

After achieving 95% accuracy in the sound detection training, the reaction time to narrow-band noise bursts (50 ms duration, centered at 2 kHz) was tested. Sound was presented at a random order (40-110 dB SPL, 10 dB step), and rats were required to poke the food dispenser within 10 seconds after initiation of sounds. Poking on either side of the food dispenser was rewarded with food pellets. A rat typically earned about 250 pellets during a test typically lasting 40 minutes. The reaction time was defined as the time between the onset time of the acoustic stimulation to the time that the rat withdrew his nose from the nose-poke. Only the trials that led to a reward were used to calculate the reaction time. A training session on the second day of the test was used to reinforce the stable operant performance.

2.4. Immunofluorescence Staining. After the behavioral test, all the rats in both groups were used for the c-Fos staining. Rats were placed in a sound attenuating booth for two hours before they were euthanized with carbon dioxide. They were then perfused transcardially with 10% phosphate formaldehyde. Their brains were taken out for postfixation in 10% formaldehyde phosphate for overnight before being transferred to a 30% sucrose in 0.1 M phosphate buffer solution (PBS) for 48 h at 4°C. For each animal, we processed a set of serial sections. Structures were delineated according to anatomical atlases [22]. Sections for the NAc were sampled from bregma 2.04 mm to bregma 1.44 mm, and the MGNs were sampled from bregma −5.76 to −6.24 mm. Coronal serial cryosections were cut to 40 μm thickness on a freezing microtome (HM 505N), and the sections (encompassing the MGN and NAc) were rinsed in 0.1 M PBS.

All the immunostaining processing was performed using free-floating sections. First, the sections were removed from the cryopreservative and rinsed in PBS. Then, the sections were blocked in blocking buffer (5% normal donkey serum, 0.3% Triton X-100 with PBS) for 30 min. The primary antibody, a rabbit anti-c-Fos antibody (diluted 1 : 300; Millipore, Temecula, CA, USA), was added to the sections and incubated overnight at 4°C on a tissue rocker. The sections were then rinsed and incubated with a donkey anti-rabbit secondary antibody labeled with Alexa Fluor 488 (diluted 1 : 300; Abcam, Cambridge, MA, US) for 2 hrs and then incubated with TO-PRO-3 iodide (1 : 500, Thermo Fisher Scientific,

Waltham, MA, US) for 20 min at room temperature. The sections were rinsed and mounted on Fisher "Superfrost" polarized slides (Fisher Scientific, Pittsburg, PA, USA), and the images were acquired with a confocal microscope (Zeiss LSM510).

2.5. c-Fos-Positive Cell Counting. Images were captured at a 63x magnification oil immersion lens with numerical aperture of 1.4. For quantitative analysis of c-Fos-positive cells, three representative images from each of three serial sections were captured. Each c-Fos-positive nucleus was counted to calculate the average number of the positive staining under double-blind conditions with the ZEN lite software 2012 (Zeiss, Germany).

2.6. Statistical Analysis. GraphPad Prism software (GraphPad Software, San Diego, CA) was used for plotting and statistical analysis. Results were presented as mean ± standard error of the mean (SEM). Student's t-tests were used for analyzing the results for ABR, reaction time, and c-Fos staining. $P < 0.05$ was taken to be statistically significant.

3. Results

3.1. ABR Results. ABR thresholds were obtained at P35from the rats in both groups. The mean ABR thresholds of the noise group ($n = 4$) were 40-50 dB higher than those of the control group ($n = 4$). The differences in the ABR threshold of the noise group and the control group were significantly different at 8, 24, and 32 kHz (Student's t-test, $P < 0.05$, Figure 1). At 2 and 4 kHz, the ABR thresholds in the noise group had no statistical difference with the control group (Student's t-test, $P > 0.05$).

3.2. Behavioral Training for Sound Detection Test. Rats in the noise group ($n = 4$) and the control group ($n = 4$) underwent operant training for sound detection after P35. A narrow band noise centered at 2 kHz was used for sound detection test to avoid the effect of hearing loss. After 3-4 weeks of training, the accurate detecting rate reached to 95%. Then, the sound reaction time was measured at different intensities (40-110 dB SPL). At the low intensities of sound stimuli (<70 dB SPL), the average reaction time of the noise group was similar to that of the control group. The sound reaction time decreased significantly when sound intensity increased. The sound reaction time of the noise group was significantly shorter than the control group at high intensities (>70 dB SPL, Student's t-test. $P < 0.05$, Figure 2). The average reaction time of the noise group ($n = 4$) was 287.1 ± 13.4 ms, 141.3 ± 18.7 ms, 107.1 ± 15.9 ms, 100.3 ± 17.3 ms, and 94.8 ± 14.9 ms at 70, 80, 90, 100, and 110 dB SPL, respectively, whereas the control group ($n = 4$) was 361.3 ± 6.6 ms, 356.1 ± 23.4 ms, 271.8 ± 24.2 ms, 194.3 ± 33.9 ms, and 177.6 ± 22.7 ms at 70, 80, 90, 100, and 110 dB SPL, respectively.

3.3. c-Fos Expression in the MGNs and the NAc. The expression of c-Fos was evaluated in frozen sections of the MGN and the NAc after the behavioral tests. The c-Fos expression in the MGN was very weak in the control group and relatively stronger in the noise group (Figure 3(a)). The difference of the number of c-Fos-positive cells in the two groups was statistically significant (Student's t-test, $P < 0.05$). c-Fos expression of the NAc in the noise group was also significantly higher than that in the control group (Figure 3(c)). The number of c-Fos-positive cells in the NAc of the noise group was significantly higher than that of the control group (Student's t-test, $P < 0.05$).

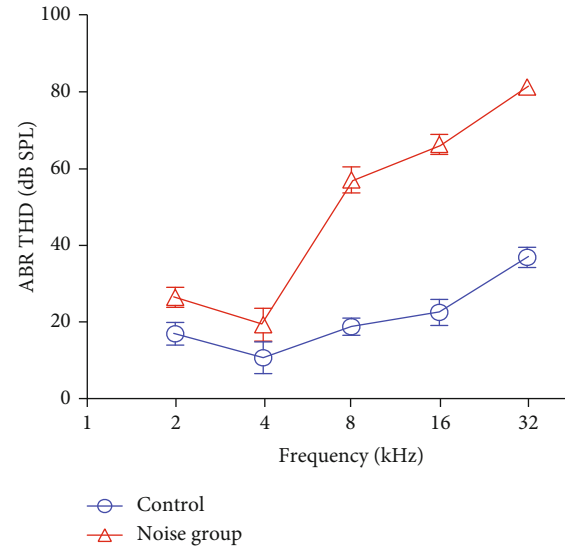

Figure 1: Thresholds of auditory brainstem response (ABR) measured from rats in the control group ($n = 4$) and the noise group ($n = 4$). The mean ABR thresholds of the noise group were significantly higher than those of the control group at 8-32 kHz, not at 2-4 kHz.

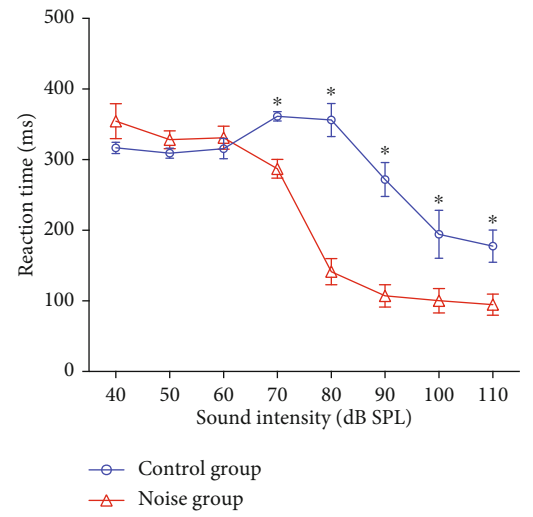

Figure 2: The reaction time-intensity functions measured from the rats in the control group ($n = 4$) and the noise group ($n = 4$) using narrow-band noise centered at 2 kHz (40-110 dB SPL, 10 dB step). At high intensity sound levels (>70 dB SPL), the reaction time in the noise group was significantly shorter than that in the control group (Student's t-test, $P < 0.05$), suggesting an increased loudness response.

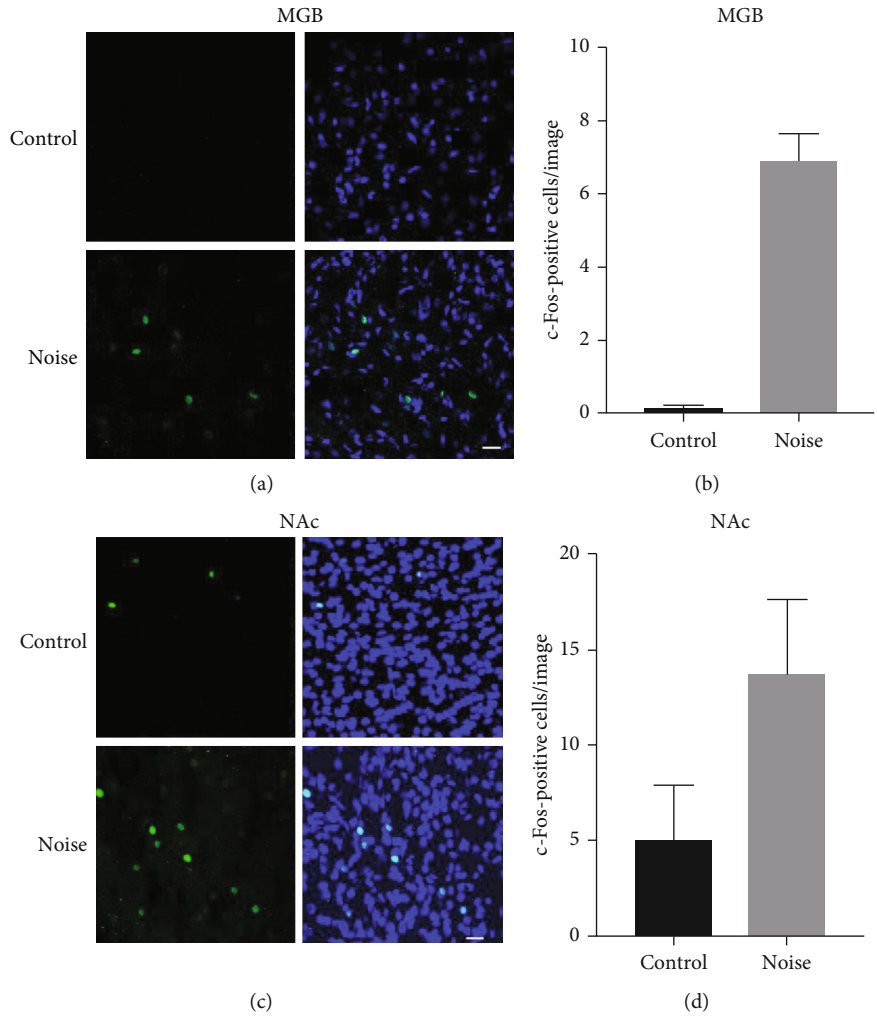

Figure 3: The c-Fos expression detected in the medial geniculate nucleus (MGN) and the nucleus accumbens (NAc) in rats. The nuclei were stained for c-Fos (green) and were visualized with TO-PRO-3 iodide (blue) (63x magnification oil immersion lens with numerical aperture of 1.4). Marker: 10 μm. (a, b) c-Fos has very few expressions in the MGN of the rats in the control group but was significantly expressed in the MGN of rats in the noise group. The c-Fos-positive cell counting showed significant deference (Student's t-test, $P < 0.05$). (c, d) c-Fos expression increased obviously in the noise group than in the control group. The number of c-Fos-positive cells of the NAc in the noise group increased significantly compared with that in the control group (Student's t-test, $P < 0.05$).

4. Discussion

In this study, we tested the effects of early-age noise exposure on sound reaction time and c-Fos expression in the NAc and the MGN in rats. We found that rats with hearing loss at an early age showed a shorter reaction time than the controls, suggesting an increase in loudness perception [23, 24]. The results suggest that early-age noise exposure may cause loudness increase at a super-threshold level [18, 20]. The rats with early-age hearing loss may perceive a louder sound perception than rats without hearing loss, consistent with hyperacusis. Our results are consistent with clinical reports that children who experienced a period of sound deprivation during childhood are more susceptible to developing tinnitus and hyperacusis [25, 26].

To detect the neural activity changes of the limbic systems that may contribute to the sound perception changes, the c-Fos stainings in the NAc and the MGN have been evaluated. c-Fos is a well-established marker to identify activated neurons in the autonomous or central nervous systems after multiple stimuli [27–29]. We found significantly upregulated c-Fos expression in the MGN and the NAc in rats with the hyperacusis-like behaviors. Our data suggested that increased excitation in the NAc and the MGN may be related with sound loudness increases after noise exposure.

The NAc, which regulates instinctive behavior and emotions, is linked to the auditory system via the MGN. The NAc projects to the MGN via different multisynaptic pathways [30]. Anatomical data indicated that the serotonergic axons from the NAc innervated the thalamic reticular nucleus (TRN) which may have a gain-control function [30, 31]. Electrical stimulation of the NAc produced mostly decreased the neural activity of the MGNs suggesting that the NAc can inhibit the activity of the auditory neurons in the MGN through TRN projections [13, 32, 33] (Figure 4). Rauschecker et al. suggested that the subcallosal areas, such as the NAc,

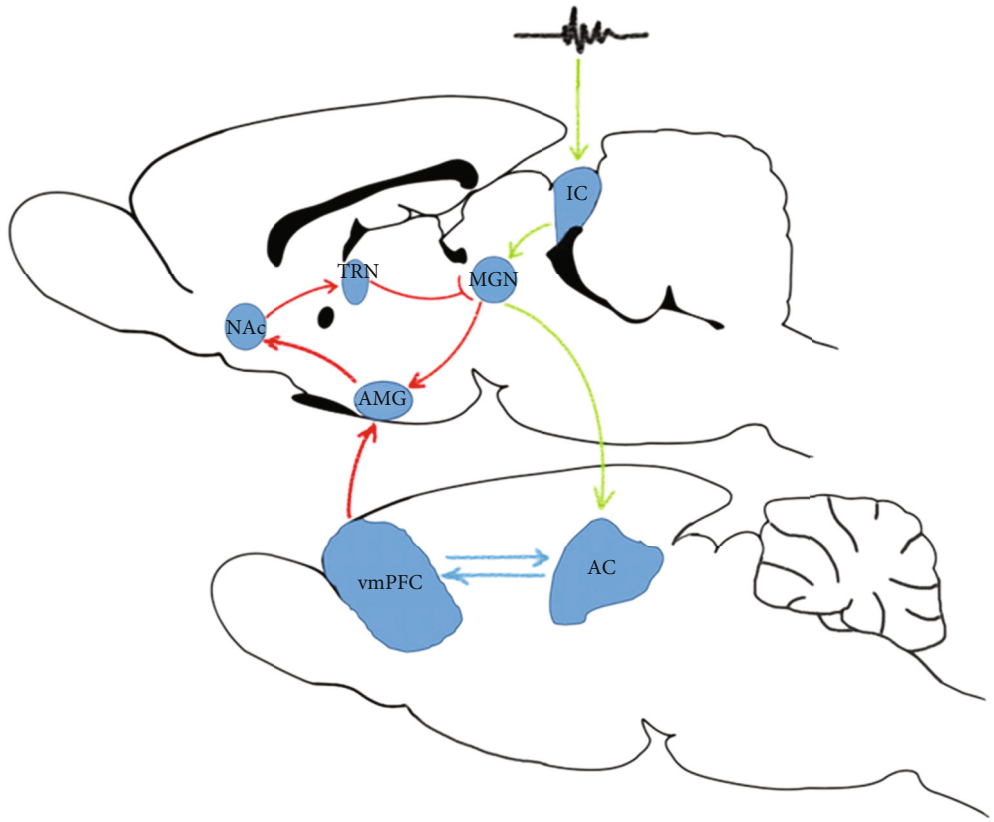

Figure 4: A schematic drawing of the neuronal structures and their projections of the auditory pathway (green lines) and limbic system (red lines) which might be involved in tinnitus and hyperacusis. Please note that the drawing primarily shows the structures and connections most relevant in the context of the proposed mechanism but are not exhaustive. Abbreviations: AC: auditory cortex; vmPFC: ventromedial prefrontal cortex; NAc: nucleus accumbens; AMG: amygdala; IC: inferior colliculi; MGN: medial geniculate nucleus, TRN: thalamic reticular nucleus.

were potentially involved in the cancellation of the tinnitus signal at the thalamic level [34]. They anticipated that tinnitus signals were generated in the auditory system, and failure to be blocked by the limbic system may lead to chronic tinnitus perception. Interestingly, in their study, they also detected hyperactivity in the NAc and the auditory cortex to the sounds at the frequency matched to the patient's tinnitus [35]. Based on their results, we predicted that failure of inhibiting NAc activity may release sound perception in the quiet (causing tinnitus) and exaggerate sound perception in the noise environment (causing hyperacusis). This may explain why tinnitus and hyperacusis are commonly presented together. In our study, increased c-Fos expression of the NAc was found in rats with hyperacusis-like behavior which supports a possible role for the NAc in modulating auditory information in hyperacusis. A recent study also found that injection of 5,7-dihydroxytryptamine (5, 7-DHT), which depleted the serotonergic projection of the NAc to the auditory system, resulted increased acoustical startle response [36]. The study suggested that the serotonergic projection of the NAc may be involved in modulating the neural activity of the MGN in processing sound signals at different intensities.

In summary, the increased neural activity in the NAc and the MGN may be related to the increased loudness perception. A failure on neural modulation between the NAc and the MGN could possibly induce tinnitus and hyperacusis. Our results suggested that early age noise exposure caused hyperactivity of the limbic circuits which may be related to increased loudness perception which is commonly seen in tinnitus and hyperacusis patients [37]. A better understanding of the NAc in hyperacusis may help us to find a novel strategy to reduce tinnitus and hyperacusis.

Data Availability

Data available on request.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Research Article

Baseline Motor Impairment Predicts Transcranial Direct Current Stimulation Combined with Physical Therapy-Induced Improvement in Individuals with Chronic Stroke

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Transcranial direct current stimulation (tDCS) can enhance the effect of conventional therapies in post-stroke neurorehabilitation. The ability to predict an individual's potential for tDCS-induced recovery may permit rehabilitation providers to make rational decisions about who will be a good candidate for tDCS therapy. We investigated the clinical and biological characteristics which might predict tDCS plus physical therapy effects on upper limb motor recovery in chronic stroke patients. A cohort of 80 chronic stroke individuals underwent ten to fifteen sessions of tDCS plus physical therapy. The sensorimotor function of the upper limb was assessed by means of the upper extremity section of the Fugl-Meyer scale (UE-FM), before and after treatment. A backward stepwise regression was used to assess the effect of age, sex, time since stroke, brain lesion side, and basal level of motor function on UE-FM improvement after treatment. Following the intervention, UE-FM significantly improved ($p < 0.05$), and the magnitude of the change was clinically important (mean 6.2 points, 95% CI: 5.2–7.4). The baseline level of UE-FM was the only significant predictor ($R^2 = 0.90$, $F_{(1,76)} = 682.80$, $p < 0.001$) of tDCS response. These findings may help to guide clinical decisions according to the profile of each patient. Future studies should investigate whether stroke severity affects the effectiveness of tDCS combined with physical therapy.

1. Introduction

Transcranial direct current stimulation (tDCS) is an emerging technique with the potential to enhance the effect of therapeutic approaches in post-stroke rehabilitation [1, 2]. According to the interhemispheric competition model [3, 4], anodal tDCS is applied to increase the excitability of the lesioned hemisphere. In contrast, cathodal tDCS is applied to decrease the excitability of the nonlesioned hemisphere. Lastly, bihemispheric tDCS involves anodal and cathodal tDCS applied simultaneously [5].

Regarding the effects of each tDCS method, it is suggested that bihemispheric tDCS has a more significant effect on chronic stroke [6–8]. Moreover, the positive effect of each tDCS approach on stroke motor recovery has been elucidated by previous studies [9–13]. Notably, recent systematic reviews reported the improvement of upper limb (UL) sensorimotor functions and improvement of activities of daily living following tDCS in post-stroke individuals [8–10, 14].

Despite its great potential, post-stroke subjects show different responses to tDCS. Furthermore, the variability of tDCS effectiveness limits its implementation as standard patient care [15]. A better understating of individual characteristics for predicting motor recovery in responding to treatment should be considered a crucial component for post-stroke rehabilitation.

Following a stroke, neural reorganization, due to spontaneous recovery or induced by therapeutic interventions, is influenced by clinical and biological factors [16–18]. Some of these factors might help to predict therapy-mediated

motor recovery [18–21], i.e., stroke chronicity [22, 23], sex [24, 25], age [23, 26], prestroke hemispheric dominance [18], and time since stroke [17].

Initial motor impairment can also predict motor outcomes [27]. Post-stroke motor recovery is highly variable [15], and individuals could present mild to severe motor impairment [28]. Overall, the initial (i.e., baseline) motor impairment is a strong predictor of functional improvement; e.g., moderate motor impairment is associated with better recovery than severe impairment in post-stroke survivors [29].

Notably, previous studies employing tDCS combined with physical therapy included patients with different motor impairment levels and reported heterogeneous results [30–32]. The variability of tDCS response could be related to different aspects related to the technique or the patient's characteristics. Regarding the tDCS, the parameters of the technique, the ideal number of sessions, and the most appropriate stimulation site (lesioned hemisphere, nonlesioned hemisphere, or both hemispheres) should be considered. Concerning the post-stroke individuals, it is important to consider the motor impairment, the location and size of the lesion, and the previous condition of the subject. The most appropriate supporting therapy should also be considered. The heterogeneous results could be related to one or more of these factors (reviewed in Simonetta-Moreau [33]).

Considering predictive factors that might guide stroke recovery, recent studies suggest the development of algorithms or models to determine functional recovery following rehabilitation in either acute or chronic post-stroke individuals [5, 34]. Although there is an increasing number of studies using tDCS in stroke rehabilitation and its relevance for clinical practice, it is unknown whether personal factors, e.g., age and sex, may predict the magnitude of the effect of tDCS on functional recovery [33]. Moreover, UL sensorimotor impairments (e.g., disrupted interjoint coordination, spasticity, and loss of dexterity) are common after stroke and persist in the chronic stage [35, 36]. These deficits may lead to decreased quality of life and social participation. Thus, this study was aimed at investigating if clinical and biological characteristics might predict the tDCS plus physical therapy effects on UL motor recovery in chronic stroke individuals. This knowledge might help to guide clinical decisions according to the clinical profile of each patient as well as to enhance clinical evidence-based practice for neurorehabilitation.

2. Methods

2.1. Design and Sample. This study is a secondary analysis of data in previously published studies [37, 38] and two ongoing studies (NCT03446378 and NCT02166619) developed at the Applied Neuroscience Laboratory (Universidade Federal de Pernambuco, Brazil).

The local ethics committee approved these studies, and all participants gave written informed consent. Each study was a double-blind (see Intervention), sham-controlled randomized clinical trial. Individuals aged >18 years were included if they presented the following criteria: (i) ischemic or hemorrhagic chronic stroke (≥3 months after onset), (ii) UL sensorimotor impairment due to stroke, and (iii) no cognitive impairment according to the Mini-Mental State Examination [39] and being able to perform some movement with the wrist and/or thumb. Exclusion criteria were as follows: spasticity at the wrist > 3 according to the Modified Ashworth Scale [40], aphasia, or any contraindications for tDCS, according to safety guidelines [41, 42]. Eighty chronic post-stroke subjects who received active tDCS treatment were analyzed.

2.2. Intervention. Participants were randomly assigned to the tDCS protocol group: anodal on the lesioned motor cortex (1 mA/13 min or 2 mA/20 min), cathodal on the nonlesioned motor cortex (1 mA/9 min or 2 mA/20 min), or bihemispheric tDCS (2 mA/20 min). The lesioned and nonlesioned motor cortex (C3/C4) was determined according to the 10/20 reference system [43]. For anodal and cathodal tDCS, the reference electrode was placed over the contralateral supraorbital area. In all trials, randomization was performed by an independent investigator not involved in any of the research phases through the website http://www.randomization.com.

All participants received ten to fifteen sessions of tDCS (3 to 5 times/week) plus usual-care physiotherapy (45 minutes to 1 hour). Physiotherapy consisted of constraint-induced movement therapy, virtual reality, or task-oriented exercises. All participants attended physical therapy sessions after tDCS. All subjects were evaluated at the baseline and after the completion of all tDCS sessions plus physical therapy (see Outcome Measurement).

Assessors (pre and post) and participants were blind to the tDCS protocol. A not-involved researcher was responsible for the application of tDCS. The allocation concealment was met using opaque sealed envelopes, which were stored in a locked room.

2.3. Outcome Measurement. The upper extremity section of the Fugl-Meyer scale (UE-FM) was used to measure sensorimotor impairment in post-stroke survivors [44, 45]. The total score ranges from 0 to 66; higher scores indicate better motor function [44]. In chronic stroke individuals, the minimal clinical important difference (mCID) ranges from 4.25 to 7.25 [46].

2.4. Data Collection. Biological (age, sex) and clinical (time since stroke, brain lesion side: dominant or nondominant according to brain dominance, determined by self-reported handedness) characteristics were collected for each participant. UE-FM scores at the baseline and after all the tDCS sessions plus physical therapy were also collected.

2.5. Statistical Analysis. Descriptive statistic was used to present clinical and biological characteristics. Data were checked for normal distribution (i.e., Shapiro-Wilk test p value > 0.05 and by visual inspection of a quantile-quantile plot).

2.5.1. Preliminary Data Analysis. Before subjecting the data to regression models, several analyses were run to control for potentially confounding baseline factors. In particular, in order to identify baseline differences between the three tDCS protocols, age, time since stroke, and UE-FM scores

were submitted to one-way ANOVAs. Chi-square (χ^2) tests were used to assess the difference between tDCS protocols for sex, handedness, and brain lesion side. To investigate the difference in the UE-FM scores at baseline and post-treatment within the entire cohort, paired Student's t-test was used, and 95% confidence intervals (CI) of mean change were reported. Finally, one-way ANOVAs were used to investigate between-group differences in UE-FM scores at post-treatment and in UE-FM changes across the three tDCS protocols. In case of significant effects, pairwise contrasts with Bonferroni corrections were used.

2.5.2. Regression Models. In order to analyze the influence of clinical and biological variables on post-stroke motor recovery, a multiple linear regression was performed. Post-treatment UE-FM was considered a dependent variable. Independent factors included in the model were variables that had previously been identified as associated with tDCS response: age and sex, time since stroke, brain lesion side, and baseline motor impairment [47, 48]. A backward stepwise regression (entry criteria: $p \leq 0.05$; removal criteria: $p \geq 0.10$) was used to find the best fit. Before performing multiple regression, independent variables were tested for multicollinearity (i.e., strong correlations among predictor variables, Pearson correlation coefficient (r) greater than 0.7), homoscedasticity, and outliers. Eighty subjects were considered an adequate sample size for regression analyses [49, 50].

SPSS version 21 (IBM, Armonk, NY, USA) was used for the statistical analysis, and the level of significance was set at $p < 0.05$.

3. Results

tDCS plus physical therapy was administrated to all participants ($n = 80$). Individuals submitted to cathodal, anodal, and bihemispheric tDCS were 34% ($n = 27$), 47% ($n = 38$), and 19% ($n = 15$), respectively. The biological and clinical characteristics of participants are presented in Table 1 (see baseline variables).

At baseline, one-way ANOVAs and chi-square (χ^2) tests showed no differences ($p > 0.05$) between the three groups for age, time since stroke, UE-FM scores, sex, handedness, and brain lesion side, respectively. Tests are presented in Table 1.

All participants showed a significant improvement in UE-FM scores after treatment ($t\text{-test}_{(79)} = 11.57$, $p < 0.001$). Moreover, the UE-FM mean change was clinically important (6.2 points, 95% CI: 5.2–7.4). Post-treatment UE-FM scores are shown in Table 1. No differences were found on the UE-FM score at post-treatment ($F_{(2,77)} = 2.732$, $p = 0.071$) and on UE-FM score changes ($F_{(2,77)} = 1.171$, $p = 0.315$), between the three tDCS protocols.

All assumptions for multiple regression were met. Stepwise regression showed that only baseline UL impairment was a significant predictor of changes in UE-FM scores after tDCS plus physical therapy ($R^2 = 0.90$, $F_{(1,76)} = 682.80$, $p < 0.001$). The results of the stepwise regression are shown in Table 2.

4. Discussion

The ability to assign the right patient to tDCS therapy would permit one to make a rational decision to add it to rehabilitation programs. Our findings showed that the baseline UL impairment might predict tDCS-induced recovery. We found significant $R^2 = 0.90$; i.e., 90% of the variance in post-treatment UE-FM scores can be predicted from the baseline UE-FM score. In particular, we found a positive regression coefficient ($\beta = 0.95$) indicating that as the value of the independent variable increases (i.e., baseline UE-FM score), the mean of the dependent variable also tends to increase (i.e., UE-FM score after treatment).

Although limited for the control group's absence, our results are in line with previous studies [10, 14, 51, 52]; i.e., tDCS plus physical therapy shows a positive effect on UL motor recovery. Moreover, we demonstrated that chronic patients reached a clinically relevant improvement after tDCS plus physical therapy regardless of tDCS protocols, age, sex, times since stroke, and brain lesion side.

This result confirms previous studies by showing that tDCS combined with other therapies induces UL recovery in patients with stroke [7, 37, 38, 53].

4.1. Predictive Factor of Recovery following tDCS. In agreement with our findings, studies [19, 26] provided evidence that initial motor impairment, commonly measured with the UE-FM, predicts functional outcomes in patients with stroke. In general, greater baseline impairment is associated with worse motor outcomes [54, 55]. However, to our knowledge, no previous study has investigated factors influencing functional UL recovery following tDCS.

One of the most common measures studied to predict UL stroke recovery is motor evoked potential (MEP) elicited with transcranial magnetic stimulation (TMS). To date, there is increasing evidence about the usefulness of TMS to study the activation and structural integrity of ipsilesional motor networks for predicting and improving motor recovery [56–59]. Indeed, studies have reported that MEP measurement had higher predictive power than clinical outcome assessment [60, 61]. However, TMS is not always available in clinical environment TMS is generally few accessible and may be influenced by several factors [62], limiting its implementation in clinical practice. Therefore, the use of clinical makers such as the Fugl-Meyer scale to predict tDCS response at the individual level might be more feasible for routine clinical use.

Future studies are needed to address the predictive power and reliability of the Fugl-Meyer scale compared with MEPs as a marker to predict motor recovery in chronic stroke following tDCS treatment. However, the prediction of tDCS responders from non-responders in chronic post-stroke individuals might be more challenging than that in the acute/subacute phase since other factors are involved, e.g., biomechanical factors [63], psychological factors, and changes in brain structural and/or functional connectivity [64]. Thus, to take into account the complexity of motor recovery in the chronic phase, predictive models should include both clinical and neurophysiological biomarkers

Table 1: Clinical and demographic characteristics.

Participant characteristics	Cathodal tDCS (9-20 min; 1-2 mA, $n = 27$)	Anodal tDCS (13-20 min; 1-2 mA, $n = 38$)	Bihemispheric tDCS (20 min; 2 mA, $n = 15$)	Between-group differences
Baseline				
Age (in years)	60.5 (±9.9)	56.6 (±.9.2)	59 (±7.8)	$F = 1.40$, $p = 0.253^{*}$
Sex, n (female/male)	27 (11/16)	38 (13/25)	15 (6/9)	$\chi^2 = 0.336$, $p = 0.845^{\#}$
Handedness, n (right/left)	27 (24/3)	38 (38/0)	15 (14/1)	$\chi^2 = 4.211$, $p = 0.122^{\#}$
Time since stroke (in months)	31.1 (±26.8)	36.7 (±28.9)	41.2 (±27.9)	$F = 0.659$, $p = 0.520^{*}$
Brain lesion side, n (dom/non-dom)	27 (16/11)	38 (20/18)	15 (7/8)	$\chi^2 = 0.652$, $p = 0.722^{\#}$
UE-FM score	27.7 (±15.7)	30.6 (±15.5)	37.9 (±11.3)	$F = 2.262$, $p = 0.111^{*}$
Post-treatment				
UE-FM score	32.9 (±15.2)	37.7 (±14.6)	43.9 (±14.2)	$F = 2.732$, $p = 0.071^{*}$

Values are mean and standard deviation, except for sex, time since stroke, and lesion side (count). tDCS: transcranial direct current stimulation; UE-FM: upper extremity Fugl-Meyer scale; dom = dominant; non-dom = nondominant. *One-way ANOVA; $^{\#}$Chi-square test.

Table 2: Results of the regression analyses.

Model	Variables	β	(SE)	β stand	t	p	R^2	R^2 change
1	Age	0.04	0.06	0.02	0.66	0.51		
	Sex	-0.24	1.18	-0.01	-0.21	0.84		
	Time since stroke	0.01	0.02	0.01	0.28	0.78		
	Brain lesion side	0.91	1.16	0.03	0.79	0.43		
	Baseline UE-FM	0.96	0.04	0.96	24.94	<0.001	0.902	0.902
2	Age	0.04	0.06	0.03	0.69	0.49		
	Time since stroke	0.01	0.02	0.01	0.31	0.76		
	Brain lesion side	0.89	1.15	0.03	0.77	0.44		
	Baseline UE-FM	0.96	0.04	0.96	25.12	<0.001	0.902	<0.001
3	Age	0.04	0.06	0.03	0.69	0.49		
	Brain lesion side	0.85	1.14	0.03	0.75	0.46		
	Baseline UE-FM	0.96	0.04	0.96	25.33	<0.001	0.901	<0.001
4	Brain lesion side	0.93	1.13	0.03	0.82	0.41		
	Baseline UE-FM	0.96	0.04	0.96	25.54	<0.001	0.901	-0.001
5	Baseline UE-FM	0.95	0.04	0.95	26.13	<0.001	0.900	-0.001

UE-FM = upper extremity Fugl-Meyer scale; SE = standard error. Note that only baseline UE-FM is a significant predictor in the regression models.

[21]. Indeed, a recent guideline and systematic review suggest that for a proper selection of post-stroke subjects for tDCS, assessment of anatomo-functional parameters and initial motor impairment should be considered [2, 65].

4.2. Nonpredictive Factors of Recovery following tDCS. Age and sex were not significant factors limiting tDCS-induced motor UL recovery. Also, previous studies have demonstrated motor recovery induced by various therapies regardless of age and sex [20, 66]. Besides, some evidence [67, 68] suggested that noninvasive brain stimulation- (NIBS-) induced plasticity is decreased with age, although some other studies are in line with our findings reporting no age-related effects [69, 70]. The tendency of elderly patients to experience more severe strokes with greater motor impairment [71] should be considered to avoid misinterpretation of aging as a predictive factor in stroke recovery. Following the same reasoning, higher frequency of severe strokes in women [24] reflecting worse motor impairment could contribute to sex-related differences in the motor outcome following NIBS. Indeed, sex differences on functional outcomes after stroke disappear after adjustment for confounding factors such as stroke severity [72].

Although our regression did not find that the brain lesion side was a significant predictor for motor recovery, a previous study found it [73]. These authors suggested that the affected UL motor recovery is dependent on brain dominance of the impaired hemisphere. Increasing evidence suggests that interhemispheric inhibition is influenced by brain dominance and in individuals with stroke is greater when the non-dominant hemisphere is affected [74]. Along with the lesion side, other factors also influence motor recovery, such

as type of stroke, lesion location (i.e., cortical or subcortical), and size [33].

Even though our first aim was to investigate predictive factors of tDCS effects on UL motor recovery in chronic stroke patients, we also reported novel findings regarding tDCS protocol comparison. Few studies have routinely investigated the bilateral (i.e., bihemispheric tDCS) versus unilateral (i.e., anodal or cathodal tDCS) similarity efficacy in changing paretic UL performance. We found no significant difference among the three tDCS protocols on UE-FM score improvement, suggesting a nondependent effect of tDCS protocol stimulation on UL recovery. In contrast, O'Shea et al. [75] have reported the superiority of anodal and cathodal over bihemispheric tDCS in speeding reaction time in chronic stroke patients. The current intensity used in our bihemispheric tDCS protocol (2 mA), or multiple sessions versus one of O'Shea et al.'s study, could explain the different findings.

Apart from the heterogeneity of tDCS parameters, the similarities seen between the tDCS groups could be related to motor impairment levels. Previous studies have suggested that individuals with mild or moderate impairment showed considerable activity in the lesioned hemisphere and/or partial integrity of the corticospinal tract [76, 77]. In light of this physiological finding, we can hypothesize that for a mild to moderate severity population, it is favorable to increase the activity present in the lesioned hemisphere, rather than inhibit the nonlesioned one. On the other hand, it is also known that patients with severe motor impairment present greater activity in the non-lesioned hemisphere [27], which could also promote negative motor-related consequences [78, 79]. Accordingly, using the cathodal tDCS to reduce the activity in the nonlesioned hemisphere could promote sensorimotor gains. Thus, the lack of difference between the three groups of tDCS might be due to different motor impairment levels across participants.

In line with our results, by comparing the effectiveness of repetitive TMS on motor recovery in relation to the time from stroke, the review of Dionísio et al. [80] also did not detect that repetitive TMS effectiveness differs among acute, subacute, or chronic phase, suggesting that time since stroke does not affect NIBS-induced effect on motor recovery. However, it is important to highlight that the time of tDCS therapy after the stroke onset could significantly influence the efficacy of a given tDCS protocol [81]. For example, based on the classical concept of interhemispheric competitive interaction (reviewed in Nowak et al. [3]), it is expected that cathodal tDCS may provide beneficial effects for some patients by reducing contralesional hemisphere activity. On the other hand, the effects may be detrimental for other subjects, depending on the individual's significance of the contralesional activity in controlling the paretic movement. This issue is still unclear and needs to be addressed in further studies.

Some limitations should be considered in this study. First, our sample size is reduced, and the results should be interpreted with caution since there is no equal distribution, considering the sex and age group. Second, our data did not include the lesion volume/site, and this could limit the interpretation of our findings since individuals with cortical or subcortical lesions could respond differently [33]. Finally, it is important to highlight that all patients underwent physical therapy, and this could influence the results since physical therapy is well established to promote motor recovery [82]. Besides, the changes in motor function may spontaneously occur after stroke. However, it is suggested that for better recovery, larger doses of physical therapy may be required to promote improvements [83]. tDCS could act as priming to enhance the effects of physical therapy [84]. Moreover, this study is a secondary analysis of previous works that showed how tDCS increased the therapy effect.

Despite the positive effects of tDCS on motor recovery [9, 10, 51], several scientific issues remain unresolved. Studies are warranted to investigate the dose-response relationship and to profile patients who might potentially benefit from tDCS.

5. Conclusion

To date, no precise indicators are available to predict positive effects following tDCS plus physical therapy on UL recovery. Our results suggest that a simple metric of baseline motor impairment by means of UE-FM may be predictive for clinical motor improvement induced by tDCS. Overall, this knowledge may help to guide clinical decisions according to the profile of each patient, reducing tDCS therapy failure and making it practically useful in clinical settings. Future studies should consider the motor impairment of post-stroke individuals to investigate personalized protocols of tDCS.

Data Availability

The data that support the findings of this study are available from the corresponding author (DP) upon reasonable request.

Conflicts of Interest

The authors report no conflicts of interest.

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Research Article

Multishell Diffusion MRI Reflects Improved Physical Fitness Induced by Dance Intervention

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Using multishell diffusion MRI and both tract-based spatial statistics (TBSS) and probabilistic tracking of specific tracts of interest, we evaluated the neural underpinnings of the impact of a six-month dance intervention (DI) on physical fitness and cognitive outcomes in nondemented seniors. The final cohort had 76 nondemented seniors, randomized into DI and control (life as usual) groups. Significant effects were observed between the DI and control groups in physical fitness measures and in attention. We detected associations between improved physical fitness and changes in diffusion tensor imagining (DTI) measures in the whole white matter (WM) skeleton and in the corticospinal tract and the superior longitudinal fascicle despite the fact that no significant differences in changes to the WM microstructure were found between the two groups.

1. Introduction

Dance intervention (DI), regardless of the type of dance, was shown to have a positive impact on the overall physical health of older adults [1]. Dancing is an activity that comprises a wide range of skills, including motor learning, action observation, sensorimotor coordination, and synchronization with a group; it engages physical endurance, balance control, motor learning, and cognitive functions [1, 2]. Keogh et al. [3] in their review concluded that dancing can significantly improve the aerobic power, lower body muscle endurance, strength, flexibility, balance, and agility of older adults. Another more recent review [4] proved the beneficial effect of dance on physical fitness (mostly reported as balance and motor skills in general) in patients with different kinds of pathology.

The influence of the different types of dance activity on cognition in healthy seniors has been tested behaviorally [5, 6]. Coubard et al. [5] reported that contemporary dance training (one lesson/week, 5.7 months) leads to improvement in switching attention in older adults. Kattenstroth et al. [6] reported positive effects of regular dancing (one lesson/week, six months) on attention in particular. Neither of these studies examined any possible neuroimaging correlates of the positive effect of DI.

Diffusion tensor imaging (DTI) is the most widely used model of diffusion MRI, which allows the evaluation of changes in the brain microstructure, especially in the white

matter (WM) [7]. Voss et al. [8] used conventional single-shell DTI and examined the effect of aerobic fitness training (three lessons/week, one year). The authors observed a relationship between increased aerobic fitness and increased fractional anisotropy (FA) in prefrontal, parietal, and temporal areas. On the other hand, they found no difference in cognitive performance between groups. The only study evaluating the effect of the DI (three lessons/week, six months) in healthy seniors with conventional DTI was performed by Burzynska et al. [9]. The authors used tract-based spatial statistics (TBSS) and selected regions of interest on the TBSS skeleton using the DTI WM atlas. They found that FA in the fornix increased in the DI group as compared to control groups. However, the change in fornix integrity did not correlate with the change in cognitive outcomes. In fact, there were no significant cognitive changes during DI surpassing changes in the control groups.

In our previous paper [10], we demonstrated subtle effects of an optimized, structured six-month dance intervention on executive functions in aged people without dementia, particularly in the Five-Point Test (FPT) which evaluates attention and executive functions [11]. In the current DTI substudy, we aimed to explore the neural correlates of the DI-induced changes in physical fitness and cognition using a multishell diffusion MRI protocol. The use of multiple shells improves the modeling of crossing fibers within each voxel [12]. Moreover, by quantifying the non-Gaussian diffusion, the multishell DTI provides more precise information about the microstructural properties of WM tissue heterogeneity [12, 13]. However, the changes in microstructural properties of the tissue derived from the multishell DTI might be caused by variety of factors, such as axonal density, axonal ordering, degree of myelination, accumulation of pathological proteins, brain atrophy, or microglial activation, and the method does not inform about distinct pathological underlying mechanisms of the observed DTI changes [14].

2. Materials and Methods

The cohort consisted of healthy senior volunteers and seniors with mild cognitive impairment (MCI), all potentially capable of participating in the intensive dance intervention. Healthy seniors were recruited using the public media such as local newspapers and radio and TV news. MCI participants were recruited from patients longitudinally followed at the First Department of Neurology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. In brief, we included subjects aged over 60 years, nonsmokers with no alcohol and/or drug abuse, and patients without serious brain injury, dementia, or major depressive disorder. For detailed information about the enrolment and randomization process, see Kropacova et al. [10]. Briefly, 120 participants were randomized to a dance intervention (DI) group and a life as usual (LAU, control) group, 60 participants in each group, using the opaque envelope method.

All subjects underwent the neuropsychological, physical fitness, and MRI examination at the baseline and after 6 months. Informed consent, in accordance with the ethics committee of Masaryk University, was obtained from each subject. The study was approved by the local ethics committee.

2.1. Dance Intervention. The dance intervention was organized by specialists from the Faculty of Sports Studies, Masaryk University, Brno, Czech Republic. The intervention took six months and included three training units (each 60 minutes) per week. The whole study lasted for three years with the DI taking place between November and April each year in small groups of up to 20 subjects. The DI program was performed at a medium physical load intensity, and subjects were supervised by an experienced tutor. The load intensity was monitored by the Borg Rating of Perceived Exertion (RPE) scale during each supervised session. The RPE is a user-friendly numerical scale that evaluates an individual's self-reported level of effort, physical exertion, and fatigue during exercise using a 15-point scale ranging from 6 (no exertion) to 20 (maximum exertion) [15]. The physical load was adjusted to the current health condition and physical fitness levels of the individual seniors, and it was kept between 11 and14 points on the RPE. The DI units included folk, country, African, Greek, and tango dancing. The choreographies were divided into smaller blocks that were gradually taught in individual lessons and modified and developed over time into the final choreography. Only subjects who completed at least 60% of the DI program were included in the final cohort [10]. The real average completion of the DI program is 78.1%.

2.2. Physical Fitness Examination. The effect of the DI was evaluated by two tests from the functional fitness assessment [16]. The 8-Foot Up-and-Go Test evaluates the agility and dynamic balance. It measures time (in seconds) required to get up from a seated position, walk 8-foot distance, return to the chair, and sit down. The lower values indicate better performance. The 30-Second Chair Stand Test evaluates lower body strength and physical endurance by measuring the number of repetitions of full stands from a chair in 30 seconds. The higher values indicate better performance.

2.3. Neuropsychological Examination. Global cognition, five cognitive domains, and activities of daily living were evaluated by complex neuropsychological testing [10]. The examination included the MoCA score [17] and individual tests from the memory domain (Taylor Figure Test [18], Wechsler Memory Scale III: Logical Memory [19]), attention domain (Wechsler Adult Intelligence Scale III: Digit Span, symbol search [20]), executive domain (Five-Point Test [11], Tower of Hanoi [21]), visuospatial domain (Taylor Figure Test [18], Judgement of Line Orientation [22]), language domain (Mississippi Aphasia Screening Test [23]), and activities of daily living (Bristol Activities of Daily Living Scale [24]). The cognitive domain Z-scores were calculated as the average Z-scores of the tests included in the particular domain [25].

Participants were classified as having MCI if they scored below -1.5 SD in at least two tests in one or more cognitive domains [25]. More specifically, we used the following criteria: MoCA $\geq$ 26 points and the score below 1.5 SD in two tests in at least one cognitive domain, MoCA $<$ 26 points

and the score below 1.5 SD in any two tests, and objective memory deficit on the MoCA and the score below 1.5 SD in at least one test from the memory domain.

2.4. DTI-MRI Examination. All subjects were scanned using the 3 T Siemens Prisma MR scanner (Siemens Corp., Erlangen, Germany) in CEITEC Masaryk University, Brno, Czech Republic, employing the following sequences: magnetization-prepared rapid gradient-echo (MPRAGE) high-resolution T1-weighted images (240 sagittal slices, slice thickness = 1 mm, TR = 2300 ms, TE = 2.34 ms, FA = 8°, FOV = 224 mm, and matrix size 224×224) and diffusion-weighted images (114 sagittal slices, slice thickness = 2 mm, TR = 9300 ms, TE = 97 ms, and FOV = 228 mm) and thirty noncollinear diffusion directions with b-values 500, 1000, and 2000 s/mm^2, ten T2-weighted acquisitions with b-value 0 s/mm^2, and three acquisitions with b-value 0 s/mm^2 with opposite polarity of phase encoding. FA and mean diffusivity (MD) were the parameters of interest.

2.5. Processing of the MRI Data. The structural connectivity of the WM was evaluated using the FSL software [26] and TBSS method [27]. Each subject's raw data was first corrected for susceptibility-induced distortions, eddy current distortions, and movements using the topup [28] and eddy [29] tools. Nonbrain voxels were excluded using the Brain Extraction Toolbox (BET) [30], and the brain extracted masks were checked one by one. Diffusion tensor at each voxel was modeled by DTIFIT function. Maps of FA and MD were calculated. The bedpostx tool was used for modeling with recommended settings [12, 31].

After preprocessing and quality control, the data underwent TBSS [27]. FA images of all subjects were nonlinearly registered to FMRIB58_FA_1mm target image and then affine transformed to the $1 \times 1 \times 1$ mm MNI152 standard space. The mean FA map was calculated, and the skeleton representing the centres of all tracts was created at the threshold 0.2. All individual FA maps were projected onto the skeleton. MD maps were processed using the information from the FA procedure. Individual maps were nonlinearly registered to the common space and projected onto the original mean FA skeleton. Mean values of DTI parameters were extracted from the final WM skeleton.

Paired differences within subjects were calculated, and a two-sample t-test design was set in a general linear model (GLM), as suggested by the FSL GLM User Guide. A randomization tool [32] with 5000 permutations was used to calculate the differences between the DI and control groups, controlled for the effect of gender, age, years of education, and the baseline MoCA score.

In addition to the whole-brain WM skeleton exploratory analysis, mean FA and MD were also computed for the corticospinal tract (CST) and the superior longitudinal fascicle (SLF) which are known to be related to motor planning and execution as well as spatial attention and speech comprehension [8, 9, 33, 34]. We used a bidirectional iterative parcellation (BIP) [35] which applies the FSL option of "probabilistic tracking with classification targets" in a bidirectional and iterative manner [35]. The method requires specific gray matter endpoint definition. Initial seed regions and inverse masks were downloaded from BIP's creator Bitbucket depository (https://bitbucket.org/dpat/). Gray matter endpoints for CST were defined as motor-sensory and brainstem. The motor-sensory endpoint mask was created using MARINA [36] by merging masks of the precentral gyrus, postcentral gyrus, and supplementary motor area. The brainstem endpoint originated in the Harvard-Oxford subcortical structural atlas and was truncated at $z = -20$ mm. The gray matter endpoints for SLF were derived from the AAL atlas [37], with the first endpoint being the angular gyrus and the second endpoint being the frontal middle gyrus. We chose specifically the angular gyrus since it is a crossmodal hub where converging multisensory information is combined and integrated to comprehend commands, manipulate mental representations, solve problems, and reorient attention to relevant information [38]. For the probabilistic tractography, the GPU version of Probtrackx was used [39]. The final tracts were thresholded at 5% probability, binarized, and masked with whole-brain WM segmentation. In order to be comparable with the methods and results of a study by Burzynska et al. [9], we additionally segmented the fornix using T1-weighted anatomical images and the FreeSurfer 6.0 (http://surfer.nmr.mgh.harvard.edu) [40] and the mri_cc function.

The longitudinal pipeline was used for preprocessing [41]. All segmentations were visually inspected, and 9 subjects (3 DI and 6 LAU) were excluded due to segmentation imprecisions. Binary masks of the fornix were created for each subject and registered to native space DWI b0 images using SPM (https://www.fil.ion.ucl.ac.uk/spm/software/spm12/). For segmentation of all tracts, see Figure 1.

2.6. Evaluation of Changes in Cognitive/Physical Fitness Measures of Interest and Changes in DTI Parameters. Equivalency in between-group baseline data was checked by chi-square tests and the Mann-Whitney test.

Mixed ANOVA and the following post-hoc tests were applied to examine DI-induced behavioral, cognitive, and DTI changes, both in the whole brain and in tracts of interest.

Paired t-tests were additionally used to test the DI-induced changes in the DI group only.

Partial correlations (Spearman correlation coefficient; MATLAB 2018) were calculated between changes in clinical measures of interest (i.e., those that revealed significant time $*$ group effects) and changes in FA and MD parameters in the whole-brain WM and above-mentioned tracts of interest in the DI group.

3. Results

Altogether, 99 (49 in the DI group and 50 in the LAU group) completed successfully the DI/LAU period. The most common reason to withdraw from the study during the DI period was an unexpected health problem of the participant or his/her partner and problems with keeping up with the time schedule of the intervention. The final research sample with good-quality clinical, cognitive, and diffusion MRI data both at the baseline and at the follow-up examination after the DI/LAU consisted of 76 participants (51 HC and 25 MCI

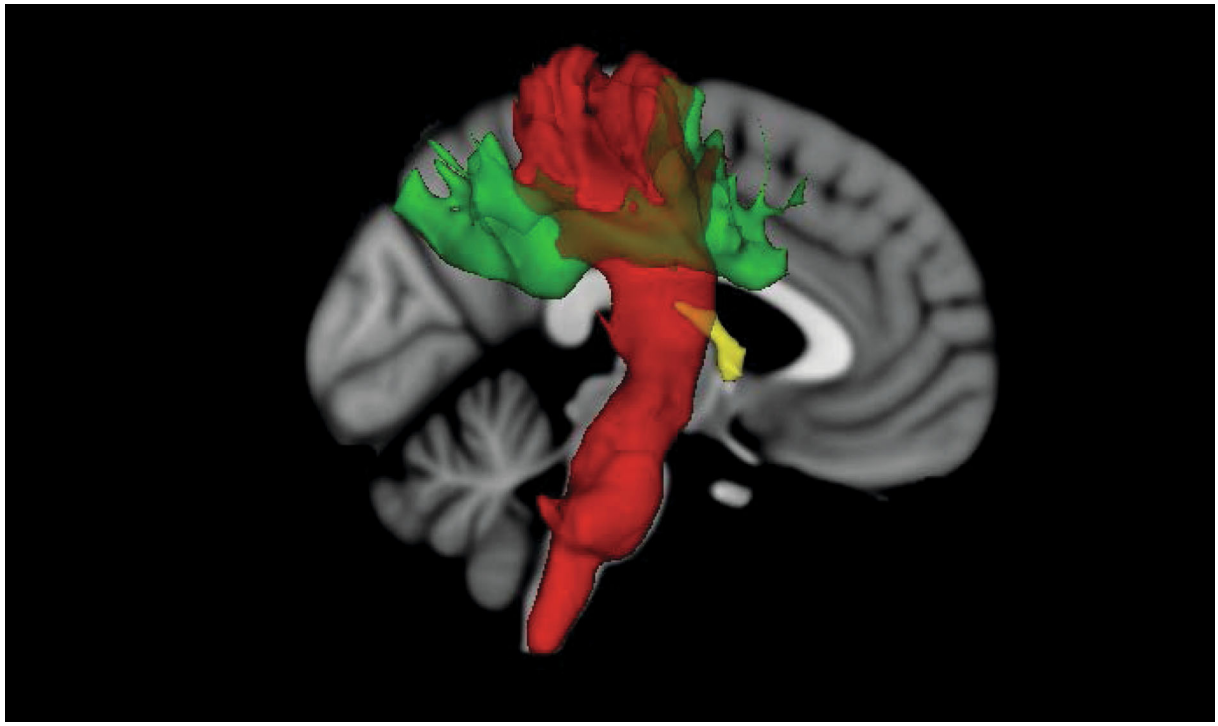

Figure 1: Visualisation of tracts of interest: red—corticospinal tract, green—superior longitudinal fasciculus, and yellow—fornix.

subjects). At the baseline, groups in the final cohort were not significantly different in age, years of education, and relative proportion of MCI participants. We found between-group differences at the baseline visit in gender and in the global cognitive MoCA score. Gender differences were due to the dropout rate, which was clearly gender-related in the DI group: fewer men than women completed the DI program. Therefore, we controlled all results for the effect of gender, age, years of education, and the baseline MoCA score. All cognitive, physical fitness, and DTI data at the baseline visit and at the follow-up visit after six months in the DI and control groups are depicted in Table 1. All cognitive, physical fitness, and DTI data at the baseline visit and at the follow-up visit after six months in both groups, divided into HC and MCI subgroups, are depicted in Supplementary Material Tables S1 and S2.

3.1. Behavioral Results. Mixed ANOVA revealed a significant time ∗ group effect (see Figure 2), in the 8-Foot Up-and-Go Test ($p = 0.006$) and in the 30-Second Chair Stand Test ($p = 0.021$). There was an effect in the attention domain ($p = 0.015$), but it did not survive the FDR correction for five cognitive domains. Complete results can be found in Supplementary Material Table S3.

Paired t-tests in the DI group revealed significant DI-induced changes (improvement) in the 30-Second Chair Stand Test ($p = 0.002$) and in the 8-Foot Up-and-Go Test ($p = 0.014$). The DI also led to improved attention ($p = 0.033$) and executive domain Z-score ($p = 0.007$) in the DI group, but the results did not survive the FDR correction for five cognitive domains. For complete results, see Supplementary Material Table S4.

3.2. DTI Results in the Whole-Brain WM and in the WM Tracts of Interest and Relation to Physical Fitness. TBSS showed no significant differences between the DI and control groups. As for the DTI measures in the subanalyses of the tracts of interest, no significant changes in FA and MD were observed between the two groups (for details, see Supplementary Material Table S3).

Partial correlation analysis showed a significant relationship between the performance in the 30-Second Chair Stand Test and global WM FA ($p = 0.016$, $R = 0.41$) in the DI group (see Figure 3).

Additional analyses in the tracts of interest in the DI group showed positive medium strength correlations between FA in the left SLF and performance in the 30-Second Chair Stand Test ($p = 0.006$, $R = 0.47$) and between MD and FA in the right CST and time to perform the 8-Foot Up-and-Go Test ($p = 0.006$, $R = 0.46$ and $p = 0.023$, $R = -0.39$, respectively). For complete results, see Supplementary Material Table S5.

4. Discussion

Our study focused on WM microstructure correlates of behavioral and cognitive effects of intensive six-month dance exercise training in mixed nondemented seniors including cognitively intact individual as well as a small proportion of MCI subjects.

We observed DI-induced effects in the physical fitness measures, namely, the 8-Foot Up-and-Go Test and 30-Second Chair Stand Test, in the DI group as compared to the control group. A better performance in these tests means improvements in the dynamic balance, agility, lower body

Table 1: (a) Descriptive characteristics. (b) Physical fitness, cognitive, and DTI data at the baseline. (c) Physical fitness, cognitive, and DTI data after 6 months. Mean ± standard deviation. *Significant difference.

	DI group	Control group	p value
(a) Descriptive characteristics			
N	37	39	—
Gender	32 F/5 M	26 F/13 M	0.042*
Age	69.3 ± 5.3	68.9 ± 6.3	0.607
Years of education	15.0 ± 2.2	15.0 ± 3.0	0.084
HC/MCI	28/9	23/16	0.121
(b) Baseline—physical fitness, cognitive, and DTI data			
8-Foot Up-and-Go Test (seconds)	5.1 ± 1.5	5.3 ± 1.4	0.890
30-Second Chair Stand Test (number of repetitions)	14.9 ± 4.0	16.8 ± 4.8	0.191
MoCA	27.4 ± 2.7	25.8 ± 2.7	0.004*
Memory (Z-score)	1.11 ± 1.01	0.93 ± 0.89	0.374
Attention (Z-score)	0.08 ± 0.58	0.02 ± 0.71	0.559
Executive (Z-score)	−0.35 ± 0.63	−0.30 ± 0.74	0.831
Visuospatial (Z-score)	0.29 ± 0.55	0.38 ± 0.56	0.417
Language (Z-score)	0.39 ± 0.46	0.40 ± 0.42	0.872
Global WM FA	0.43 ± 0.02	0.42 ± 0.02	0.979
Global WM MD (mm^2s^{-1})	0.0007 ± 0.00002	0.0007 ± 0.00005	0.230
(c) After 6 months—physical fitness, cognitive, and DTI data			
8-Foot Up-and-Go Test (seconds)	4.8 ± 1.2	5.6 ± 2.3	0.537
30-Second Chair Stand Test (number of repetitions)	16.4 ± 4.1	16.2 ± 6.3	1.000
MoCA	27.0 ± 2.7	26.6 ± 2.5	0.367
Memory (Z-score)	1.30 ± 0.77	1.15 ± 0.92	0.318
Attention (Z-score)	0.26 ± 0.71	−0.06 ± 0.75	0.046*
Executive (Z-score)	−0.02 ± 0.82	−0.22 ± 0.65	0.278
Visuospatial (Z-score)	0.30 ± 0.59	0.48 ± 0.55	0.166
Language (Z-score)	0.46 ± 0.43	0.42 ± 0.51	0.826
Global WM FA	0.43 ± 0.02	0.43 ± 0.02	0.751
Global WM MD (mm^2s^{-1})	0.0007 ± 0.00002	0.0007 ± 0.00005	0.238

strength, and physical endurance. These parameters are key factors in preserving mobility and independence in older age [16]. A positive effect of DI on physical fitness was consistently reported by others and was summarized in a review by Hwang and Braun [1]. On the other hand, the effects of DI on cognition are more heterogeneous and have remained inconsistent. Some authors reported a positive DI-induced effect on attention [5, 6, 42, 43] and memory [42, 43] functions, but positive changes were reported also in groups who underwent conventional fitness training [42, 43]. In this DTI substudy, we observed a DI-induced effect solely on the attention domain, but this result did not survive the FDR correction and therefore should be interpreted with caution. We did not observe any DI-induced effects between both groups in the microstructure in the WM skeleton or in tracts of interest.

Our major study aim was to identify whether WM microstructural changes, as assessed by FA and MD measures derived from a multishell diffusion protocol, may underlie dance-induced behavioral improvements. FA is a directionally dependent sensitive marker of microstructural changes (e.g., myelination, fiber orientation, and axonal diameter) [14]. Increase in FA can be explained by, e.g., higher packing density or increased directional organization of axons and/or stabilization or increase in myelin integrity [14, 44]. MD is a directionally independent measure that describes overall diffusion within the voxel [45]; increased MD is characteristic for regions where neural microstructures (e.g., axonal cell membranes, myelin sheaths, and neurofilaments) are displaced by intra- and extracellular water [46]. Studies showed that the increase in MD and decrease in FA are the common process in healthy aging [47]. The WM integrity also reflects age-related variability in cognitive outcomes in healthy aged individuals [48] such that increased FA/decreased MD relates to increased cognitive performance [46].

A positive relationship between improved physical fitness and increased WM integrity in this study was detected in the

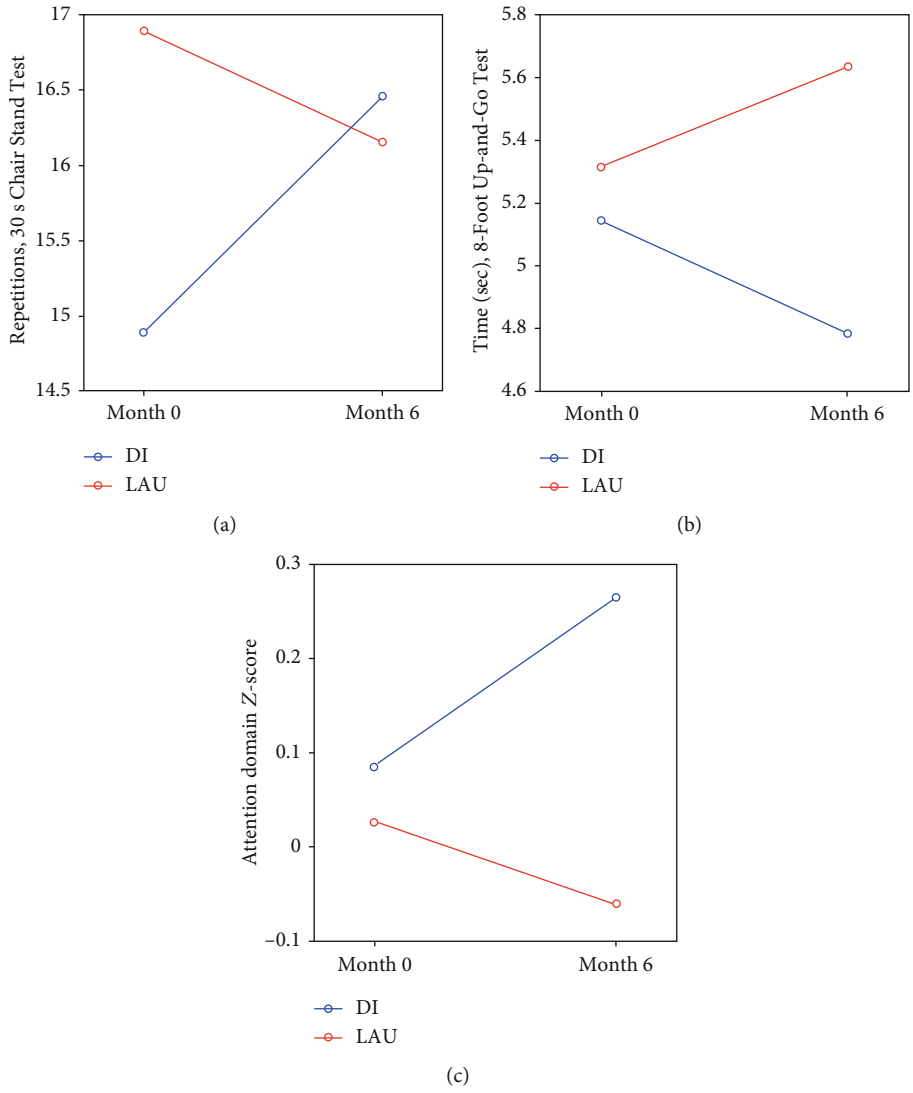

Figure 2: Mixed ANOVA results: significant time ∗ group changes. DI: dance intervention; LAU: life as usual. (a) Number of repetitions, 30-Second Chair Stand Test (increase means improvement); (b) time, 8-Foot Up-and-Go Test (decrease means improvement); and (c) attention domain Z-score.

whole brain as well as in the WM tracts of interest related to motor learning and movement execution, as well as to spatial attention, manipulation of mental representations, and speech comprehension. The CST projects from the motor cortices to the spinal cord and plays a key role in the control of voluntary movement [34]. The SLF is considered to be the major cortical association fiber pathway. As for the particular results of correlation with increased physical endurance, the SLF plays a role in regulating motor learning and higher aspects of motor behavior [33, 34]. Based on the results of Burzynska et al. [9], we also focused on the fornix, the major efferent tract of the hippocampus with a key function of memory formation and consolidation [49], although engagement in motor functions in normal aging has also been described [50]. Unlike Burzynska et al. [9], we did not observe any relationship between changes in DTI measures within this tract and cognitive functions or physical fitness.

Our results were similar to those of Voss et al. [8] who did not find significant DTI changes on a group level even after a whole year of the aerobic intervention (walking performed three times per week). The authors observed that increased FA in the WM of prefrontal, parietal, and temporal areas (no tracts were specified in this work) was related to changes in cardiorespiratory fitness measures in the walking intervention group. However, significant associations were not supported by the TBSS analysis. No specific tracts were analysed.

Some novel methodological aspects of the current study should be highlighted: we used the multishell diffusion MRI protocol which is thought to surpass conventional single-shell DTI in terms of its ability to accurately evaluate microstructural properties with varied restrictions to diffusion [13]. In addition to TBSS, tracts of interest were delineated including the probabilistic tracking method applicable for evaluating long association/projection pathways [35]. However, despite

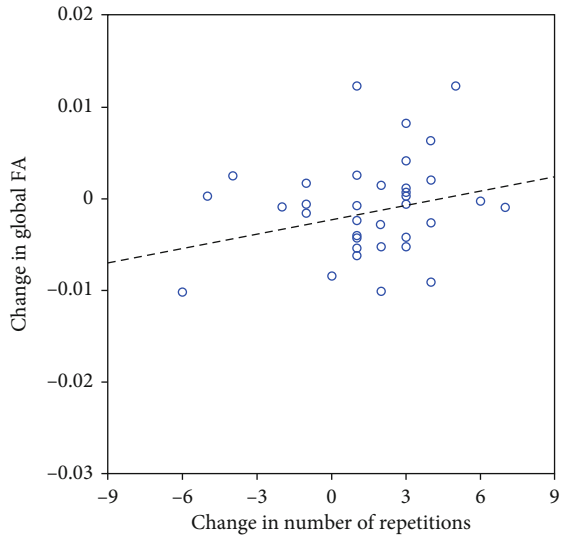

Figure 3: Relationship between change in FA and change in performance in the 30-Second Chair Stand Test.

using these methods, we were not able to identify changes in WM integrity due to the DI.

It would have been interesting to examine the HC and MCI subjects separately. However, this was not possible due to a small proportion of MCI subjects in DI and LAU cohorts.

5. Study Limitations

The distribution of demographic and cognitive characteristics and the number of MCI subjects in both groups were comparable in the whole cohort of 99 participants [10]. However, some subjects had to be discarded because of incomplete or low-quality diffusion MRI data. This led to rather disproportional distribution of MCI subjects in the groups of this DTI substudy although the number of MCI subjects in the DI and LAU groups was not significantly different.

The number of MCI patients in DI and LAU groups was too low to perform separate analysis for MCI.

The MoCA score was lower in the LAU group as compared to the DI group. Despite the fact that we controlled for the effect of the baseline MoCA score in our further analyses, we cannot fully exclude a possible effect of unequally distributed MCI participants in both groups. Another limitation of the study is a rather low number of applied diffusion directions for probabilistic tractography analysis.

6. Conclusion

In conclusion, we showed that 6 months of intensive DI can increase physical fitness measures evaluating lower body muscle endurance, agility, and balance in aged nondemented individuals and is associated with the enhancement in structural integrity, particularly in specific tracts that are engaged in motor behavior, regulation of motor learning, and coordination and control of voluntary movement. Future studies should focus on possible differences in the behavioral effects of DI and related DTI changes separately in the groups of heathy seniors and MCI patients.

Data Availability

Anonymised imaging data of this study will be available on reasonable request from any qualified researcher, following the EU General Data Protection Regulation.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

We thank Anne Johnson for English language editing.

Acknowledgments

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Supplementary Materials

Table S1: DI group and HC and MCI subgroups; mean ± standard deviation. Table S2: LAU group and HC and MCI subgroups; mean ± standard deviation. Table S3: mixed ANOVA—behavioral, cognitive (FDR-corrected), and DTI (FDR-corrected) results. *Significant. WM: white matter; CST: corticospinal tract; SLF: superior longitudinal fasciculus; FA: fractional anisotropy; MD: mean diffusivity. Table S4: paired *t*-tests: DI-induced changes in the DI group—behavioral, cognitive (FDR-corrected), and DTI (FDR-corrected) results; mean ± standard deviation. *Significant. WM: white matter; CST: corticospinal tract; SLF: superior longitudinal fasciculus; FA: fractional anisotropy; MD: mean diffusivity. MD unit (10^{-3} mm^2 s^{-1}). Table S5: partial correlations (Spearman correlation coefficient; MATLAB 2018) between changes in clinical measures of interest (i.e., those that revealed significant time ∗ group effects) and changes in FA and MD parameters in the WM in the DI group; *p* value/correlation coefficient. *Significant. WM: white matter; CST: corticospinal tract; SLF: superior longitudinal fasciculus; FA: fractional anisotropy; MD: mean diffusivity. *(Supplementary Materials)*

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Research Article

Electroacupuncture Improves Cognitive Function in Senescence-Accelerated P8 (SAMP8) Mice via the NLRP3/Caspase-1 Pathway

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Background. Clinically, electroacupuncture (EA) is the most common therapy for aging-related cognitive impairment (CI). However, the underlying pathomechanism remains unidentified. The aims of this study were to observe the effect of EA on cognitive function and explore the potential mechanism by which EA acts on the NLRP3/caspase-1 signaling pathway. *Main Methods*. Thirty male SAMP8 mice were randomly divided into the model, the 2 Hz EA and 10 Hz EA groups. Ten male SAMR1 mice were assigned to the control group. Cognitive function was assessed through the Morris water maze test. Hippocampal morphology and cell death were observed by HE and TUNEL staining, respectively. The serum IL-1β, IL-6, IL-18, and TNF-α levels were measured by ELISA. Hippocampal NLRP3, ASC, caspase-1, GSDM-D, IL-1β, IL-18, Aβ, and tau proteins were detected by Western blotting. *Key Findings*. Cognitive function, hippocampal morphology, and TUNEL-positive cell counts were improved by both EA frequencies. The serum IL-1β, IL-6, IL-18, and TNF-α levels were decreased by EA treatment. However, 10 Hz EA reduced the number of TUNEL-positive cells in the CA1 region and serum IL-1β and IL-6 levels more effectively than 2 Hz EA. NLRP3/caspase-1 pathway-related proteins were significantly downregulated by EA, but 2 Hz EA did not effectively reduce ASC protein expression. Interestingly, both EA frequencies failed to reduce the expression of Aβ and tau proteins. *Significance*. The effects of 10 Hz EA at the GV20 and ST36 acupoints on the NLRP3/caspase-1 signaling pathway may be a mechanism by which this treatment relieves aging-related CI in mice.

1. Introduction

Cognitive impairment (CI) is a common neurological disease among the elderly [1]. With the rapid aging of the global population, the proportion of patients with CI has been increasing year by year [2]. Current studies have found that the prevalence of dementia is 1% in people over 60 years old and more than 40% in people over 85 years old [3, 4]. Although the exact pathogenesis of CI is not yet clear, hippocampal pyroptosis induced by the chronic inflammatory cascade has been proposed by many scholars [5–7].

Pyroptosis is a new mechanism of cell death discovered in recent years. Caspase-1-mediated cell pyroptosis is a classical pathway that can be caused by chronic inflammation in aging [8]. Caspase-1-mediated cell pyroptosis is accompanied by the release of a large number of proinflammatory factors, which induces a cascade of amplified inflammatory responses, and staining reveals that the nuclear DNA undergoes changes similar to those that occur in cell apoptosis [9]. The major difference between caspase-1-mediated cell pyroptosis and cell apoptosis is that in the former process the cell membrane is destroyed, the cell swells due to increased

permeability, and the contents of the cell are released to the extracellular environment [9, 10]. Furthermore, NOD-like receptor protein 3 (NLRP3), apoptosis-associated speck-like protein containing a CARD (ASC), and cysteinyl aspartate-specific protease-1 (caspase-1) are activated, forming the NLRP3 inflammasome [11]; additionally, the production of interleukin- (IL-) 1β and IL-18 is induced [12]. Then, the downstream signaling pathways are activated, promoting inflammation and inducing neural plasticity damage and other neuronal damage [13].

Electroacupuncture (EA) is commonly used as a clinical rehabilitation therapy to improve cognitive dysfunction. Two main EA frequencies are commonly used, namely, low frequency (2 Hz) and high frequency (10 Hz), both of which can effectively improve indexes of clinical outcomes [14]. EA can effectively reduce the levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), and tumor necrosis factor-α (TNF-α), helping to inhibit the inflammatory response in a variety of neurological diseases [15–17]. This study is aimed at revealing the potential mechanism by which different frequencies of EA improve cognitive function by inhibiting pyroptosis of the hippocampus in SAMP8 mice; the underlying goal is to provide new therapeutic ideas for a rational selection of EA therapy as an intervention in clinical and basic research on cognitive impairment.

2. Materials and Methods

2.1. Animals and Ethics Statement. Seven-month-old male senescence-accelerated P8 (SAMP8) mice and senescence-resistant R1 (SAMR1) mice were purchased from the experimental animal center of the First Affiliated Hospital of Tianjin University of Chinese Medicine (Tianjin, China). The experiment was conducted in accordance with the laboratory animal use regulations of the Animal Care and Use Committee of Heilongjiang University of Chinese Medicine. All animals were housed in a specific-pathogen-free room (20~22°C, 40%~60% humidity) under a 12 h day/night cycle with sterile feed and autoclaved water ad libitum. After 7 days of adaptation to the new environment, all animals underwent the formal experiment.

2.2. Animal Grouping and Administration. Ten SAMR1 mice (7 months old, 22~25 g) were assigned to a control group ($n = 10$; day 0) that received no intervention. Thirty SAMP8 mice with CI (7 months old, 22~25 g) were randomly divided into three groups ($n = 10$ each; days 0, 1): a model group, which received no intervention; (2) a low-frequency EA group, which received EA (1 mA, 2 Hz) for 30 min once daily for 14 consecutive days; and (3) a high-frequency EA group, which received EA (1 mA, 10 Hz) for 30 min once daily for 14 consecutive days. Seven days before grouping and interventions, all mice underwent Morris water maze (Biobserve, Bonn, Germany) cued training tests to select for mice with CI. The criterion for selecting mice with CI was a significantly prolonged escape latency (>80 s) according to our previous study [18]. After the EA intervention ended, the mice were tested with the Morris water maze to evaluate the cognitive ability of each group. Samples of hippocampal tissue and serum were collected for further evaluation after cognitive function assessment. Neuropathological staining was performed by HE staining, and dead cells were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Enzyme-linked immunosorbent assays (ELISAs) were used to detect the serum levels of IL-1β, IL-6, IL-18, and TNF-α. Western blotting was used to detect the expression levels of NLRP3, ASC, caspase-1, GSDM-D, IL-1β, IL-18, tau and Aβ (Figure 1).

2.3. EA Treatment. The mice from the two EA groups were fixed in a prone position with a stereotaxic device. The acupuncture needles (0.3 mm diameter, Guizhou Ande Medical Appliances, Ltd.) were inserted at a depth of 2-3 mm into the Baihui acupoint (GV20) and Zusanli acupoint (ST36), and then, a Great Wall Acupoint Nerve Stimulator (Model: KWD-808I, Changzhou Wujin Great Wall Medical Equipment Co., Ltd., Changzhou, China) was used. The control and model groups did not receive any EA treatment.

2.4. Cognitive Function Assessment. Cognitive function was assessed 7 days before EA administration by the Morris water maze test to select for mice with CI. The Morris water maze comprises two pieces of equipment: a circular pool and an automatic video analysis system for movement tracking and recording. The circular pool had a diameter of 200 cm and a height of 80 cm and was divided into four black quadrants. Different color stickers were placed above the inner walls of different quadrants. All animals performed 4 trials/day with 10 min intertrial intervals and a maximum trial duration of 90 s. Each mouse was allowed to remain on the platform for 30 s at the end of each trial, and a visible circular platform was placed in a different quadrant 1.5 cm above the water for each trial. The criterion for selecting mice with CI was a significantly prolonged escape latency (>80 s) before animal grouping and EA administration according to our previous study [18].

After EA administration ended, cognitive function was assessed in each group mice once per day for six consecutive days. This test was mainly divided into two aspects: (1) a navigation experiment conducted on the first five consecutive days to measure the learning ability of the mice during which each group of mice was placed into the water at a fixed position in the first quadrant, and the position and movement track of the mice in the water were recorded in real time with a high-speed camera. The assessment indexes were escape latency (seconds), the distance travelled (mm), speed (mm/s), and the time required for the mice to find and climb onto the platform. The maximum swimming time was set at 120 seconds. The time to reach the platform, travelling distance, and speed were observed and recorded. If an animal did not find the platform within 120 seconds, it was led to the platform, and the escape latency was recorded as 120 seconds. Then, the distance travelled and speed were recorded. (2) A space exploration experiment conducted on the 6th day to measure the ability of the mice to maintain long-term memories during which the platform was removed, and the number of times that the mice crossed the location where the platform had been was calculated as the

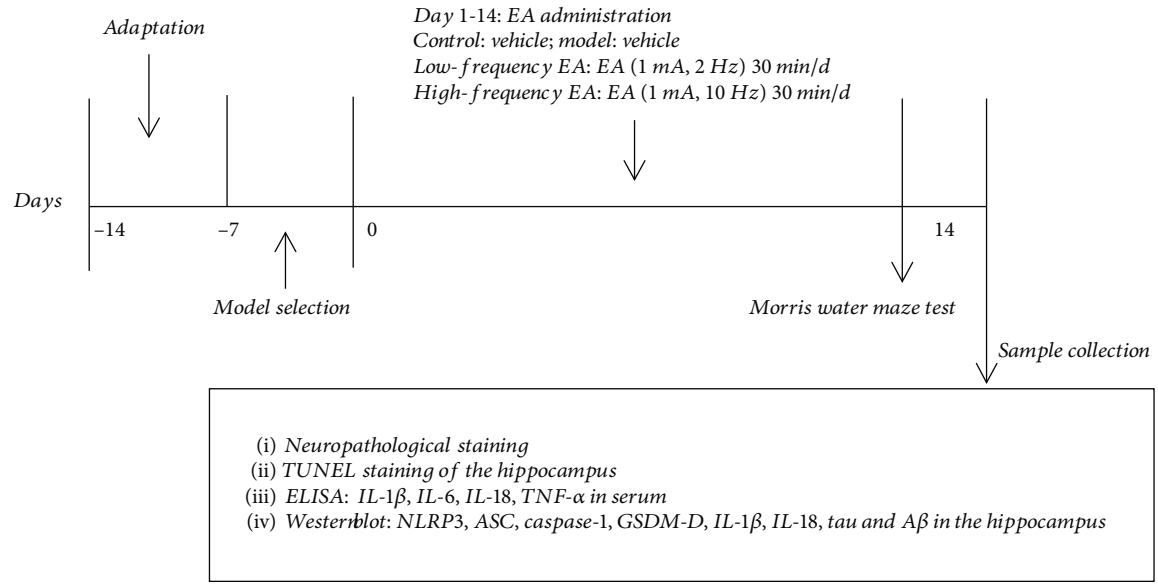


Figure 1: Experimental procedures.

assessment index. During the experiment, all groups, including the control group, underwent the above tests. The laboratory environment was kept quiet, the water temperature was maintained at 22~26°C, and the light and objects around the water maze pool remained unchanged to reduce experimental errors caused by interference from the external environment. After each experiment, the pool was cleaned, the hair of the mice was dried, and the mice were given free access to food and water in accordance with the ethical requirements of animal welfare guidelines.

2.5. Neuropathological Staining. The mice were anesthetized with isoflurane gas anesthesia using a small animal anesthesia machine (Shanghai Sango Biotechnology Co., Ltd., Shanghai, China) after the final Morris water maze assessment. The brain tissues of the mice were placed in phosphoric acid buffer (PB) containing 4% paraformaldehyde and then fixed for 24 h. The tissues were embedded in paraffin and cut into continuous coronal sections. The slices were routinely dewaxed with dimethyl benzene and then rehydrated with the following alcohol series: xylene (I) 5 min, xylene (II) 5 min, anhydrous ethanol (I) 2 min, anhydrous ethanol (II) 2 min, 95% ethanol (I) 2 min, 95% ethanol (II) 2 min, and 80% ethanol 1 min. The slides were washed with distilled water for 1 min. Hematoxylin stain was applied for 5 min and rinsed away with tap water for 1 min. Next, 1% HCl ethanol was applied for 3 s for differentiation, and the samples were washed for 2 s; blue (with warm water or 1% ammonia, etc.) was applied for 10 s followed by a water rinse for 1 min, rinsing with distilled water for 1 min, and staining with 0.5% eosin for 2 min. Conventional dehydration, clearing, and sealing were performed. We selected 1 slice at intervals of 10 slices, the slice thickness was 4 μm, and a total of 10 slices were selected for observation. Microscopy (OPTEC, BK-DM320/500, Germany) at 400, 800, and 1600x magnification was used to observe and photograph changes in the pathological structure of the hippocampal CA1 and CA3 regions.

2.6. TUNEL Staining. Paraffin sections of hippocampal tissues were washed with PBS, and the sections were placed in a DNase-free protease K solution of 20 g/mL and incubated at room temperature for 30 min. The specimens were washed with PBS for 5 min 3x, and then, 3% H_2O_2 solution was added for 10 min of room temperature incubation to eliminate endogenous peroxidase activity. The sections were permeabilized in PBS solution containing 0.1% Triton X-100 for 15 min. Then, a TUNEL detection solution containing TdT enzyme and biotin was drizzled onto the section and incubated in darkness at 37°C for 60 min. After the tissue was washed with PBS, the stop solution was applied dropwise to the sections, which were then incubated at room temperature for 10 min. The sections were washed with PBS for 5 min 3x; then, streptavidin-HRP working fluid was applied dropwise, and the sections were incubated at room temperature for 30 min. After 3 washes in PBS for 5 min each, DAB chromogen was applied dropwise to the sections, and they were incubated at room temperature for 10 min. The slices were washed with PBS for 5 min 3x, and nuclear staining was conducted with hematoxylin staining solution. Finally, the specimens were washed with PBS for 5 min 3x and sealed for observation. Five uncrossed and repeated fields were selected from each pathological section under an optical microscope, and cells with brown-yellow particles in the cytoplasm were regarded as positive apoptotic cells. We selected 1 slice at intervals of 10 slices, the slice thickness was 4 μm, and a total of 10 slices were selected for observation. Ten fields per image were randomly selected to calculate the mean number of TUNEL-positive cells. ImagePro Plus 6.0 pathological image analysis software (Media Cybernetics, Inc., Rockville, MD, USA) was used to calculate the number of TUNEL-positive cells, and the average value was taken as the number of positive cells in the sample: TUNEL – positive cells ratio (%) = number of positive apoptotic cells/(number of positive apoptotic cells + number of negative apoptotic cells) × 100%.

2.7. ELISA. At the end of the experiment, eyeballs were removed for blood collection. The serum was separated by centrifugation at 3300 r/min at 4°C for 10 min, stored in a refrigerator at -80°C, and then used to measure the serum IL-1β, IL-6, IL-18, and TNF-α levels. Using an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), the IL-1β, IL-6, IL-18, and TNF-α levels were measured following the manufacturer's instructions.

2.8. Western Blotting. The frozen mouse hippocampal tissue in the -80°C refrigerator was taken out and thawed. The hippocampus was separated, transferred to an Eppendorf tube, and cut into pieces as much as possible with special scissors, and then, 50 mg of brain tissue was mixed with 300 μL of RIPA lysate according to the instructions of the protein extraction reagent kit (Elabscience Biotechnology Co., Ltd., Wuhan, China). Then, 50 μg of protein from each group was transferred to PVDF membranes (Thermo Fisher Scientific) and blocked with 5% nonfat milk overnight at 4°C. After washing, the membranes were incubated with the following primary antibodies: NLRP3 (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), ASC (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), GSDM-D (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), Caspase-1 (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), IL-1β (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), IL-18 (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), Tau (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA), and Aβ (rabbit polyclonal, 1 : 1000, Cell Signaling Technology, Danvers, MA, USA). The membranes were subsequently sealed for incubation with the antibodies. AB luminescent solution (Beijing Priilet Co., Ltd., Beijing, China) was used for development and exposed onto the imaging system (Kodak, Rochester, NY, USA). Strip gray scale analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and seven samples in each group.

2.9. Statistical Analysis. SPSS 22.0 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA) software were used for statistics and mapping. Data were presented as the mean ± standard deviation. All data were collected and analyzed in a blinded manner. Using one-way analysis of variance (ANOVA), two-way ANOVA or two-way repeated-measures ANOVA followed by Bonferroni's post hoc test, we analyzed the escape latency, distance travelled, swimming speed, and target platform crossing number in the Morris water maze test. The Student-Newman-Keuls test was also used for multiple comparisons. Student's t-test (two-group comparison) was performed for intergroup comparisons under the condition of a normal distribution and homogeneity of variance. A nondifferential test was used when the variances were uneven. $P < 0.05$ was considered statistically significant.

3. Results

3.1. EA Treatment at the GV20 and ST36 Acupoints Improved Cognitive Function in SAMP8 Mice. We applied two different frequencies of EA treatment to SAMP8 mice for 14 days to determine whether stimulation at the GV20 and ST36 acupoints can protect against cognitive dysfunction. Two aspects of cognitive function were assessed (Figures 2(a)–2(e)). Significantly prolonged escape latency and a longer distance travelled were observed for the SAMP8 mice in the model group compared with those in the control group ($P < 0.01$). The escape latency and distance travelled of the SAMP8 mice were significantly decreased after 14 days of EA treatment at both frequencies ($P < 0.01$). No direct evidence indicated the frequency at which EA more effectively reduced escape latency and the distance travelled ($P > 0.05$) (Figures 2(a) and 2(b)). Typical swimming trajectories of the mice in each group in the first five days are shown in Figure 1(e). During the experiment, no significant difference in swimming speed was observed between each group ($P > 0.05$) (Figure 2(c)), indicating that the test frequency did not cause exhaustion and that the motor function of the model animals was not damaged. On the other hand, we found that compared with the control group the number of target platform crossings in the model group decreased significantly ($P < 0.01$), and 14 days of consecutive EA treatment could effectively improve the crossing times of the SAMP8 mice. No direct evidence indicates the frequency at which EA was more effective ($P > 0.05$) (Figure 2(d)).

3.2. EA Treatment at the GV20 and ST36 Acupoints Alleviated Hippocampal Neuropathological Injury in SAMP8 Mice. HE staining showed that the neurons in the hippocampal CA1 and CA3 regions of SAMR1 mice in the control group had complete, clearly, and orderly structures, and no abnormalities were observed. In the model group, pyramidal cells in the hippocampal CA1 and CA3 regions were sparse, and the gaps increased. Obvious pathological, morphological, and structural changes (black arrow) were mainly observed: cell boundaries were unclear, cell body swelling increased, and nuclear shrinkage migrated. The pathological changes in neurons in the hippocampal CA1 and CA3 regions in the 2 Hz EA and 10 Hz EA groups were alleviated to a certain degree compared with those in the model group (black arrow), and the effects were the most significant in the 10 Hz EA group, with an orderly arrangement of neurons, clear cell boundaries, and a small number of morphological and structural abnormalities of neurons (black arrow) (Figure 3).

3.3. EA Treatment at the GV20 and ST36 Acupoints Reduced Cell Death in the Hippocampal Neurons of SAMP8 Mice. TUNEL-positive cells were identified by a brown-yellow lesion structure (black arrow) (Figure 4(a)). The number and ratio of TUNEL-positive neurons in the hippocampal CA1 and CA3 regions in the model group were significantly increased compared with those in the control group (Figures 4(b) and 4(c)) ($P < 0.001$), and the number and ratio of TUNEL-positive neurons in the hippocampal CA1 and CA3 regions in the 2 Hz EA and 10 Hz EA groups were significantly decreased compared with those in the model group (Figures 4(b) and 4(C)) ($P < 0.01$). Compared with EA at

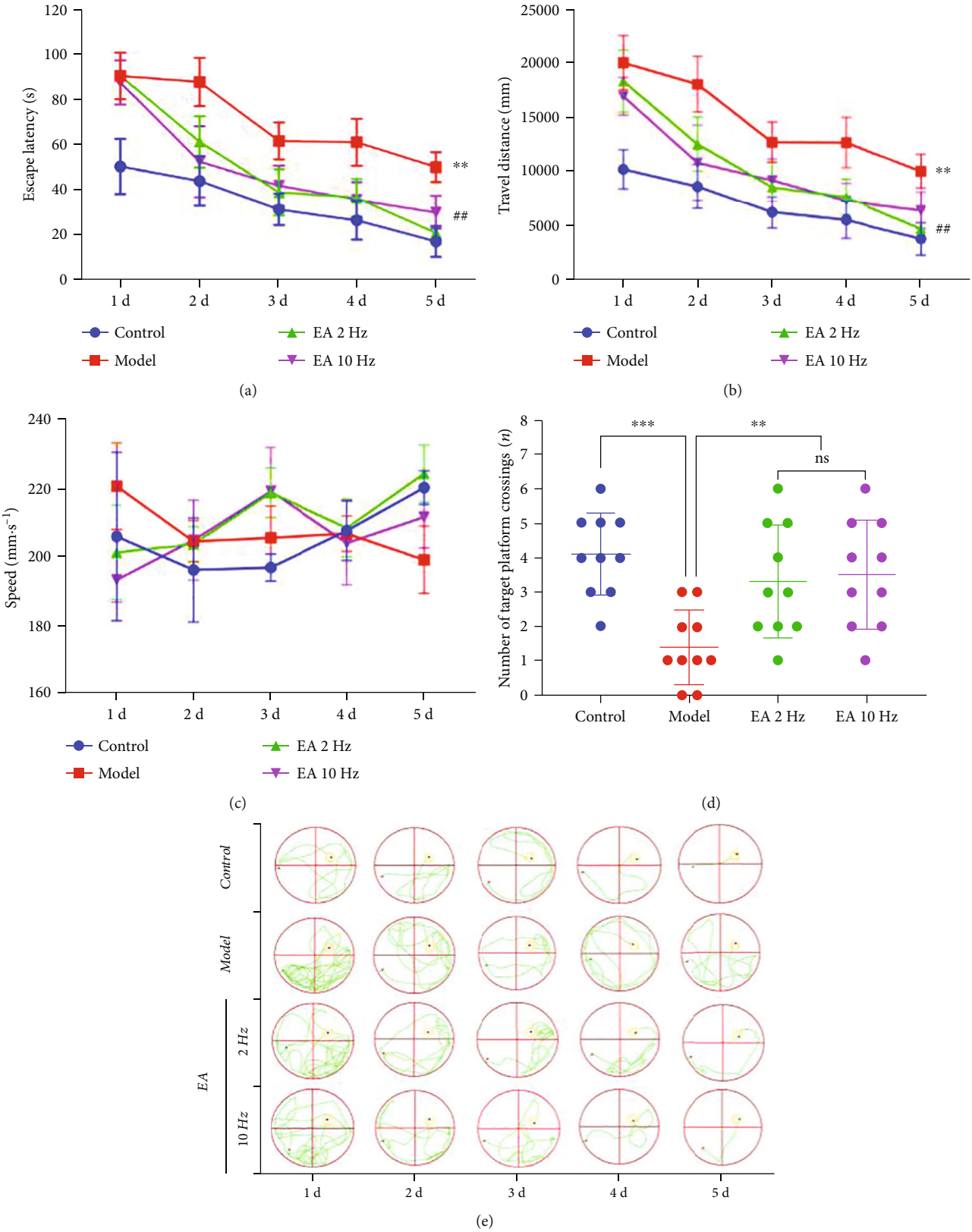


Figure 2: Electroacupuncture (EA) treatment enhanced learning and memory abilities in SAMP8 mice in two different cognitive function assessment experiments. (a) The escape latency in the navigation experiment. (b) The distance travelled in the navigation experiment. (c) The speed in the navigation experiment. (d) The target platform crossing number in the space exploration experiment. (e) The typical swimming trajectories in the navigation experiment. The data are shown as the mean ± SD of 10 mice per group. $^{***}P < 0.001$ and $^{**}P < 0.01$ vs. the model group; $^{\#\#}P < 0.01$ vs. the 2 Hz EA group. ns: not significant.

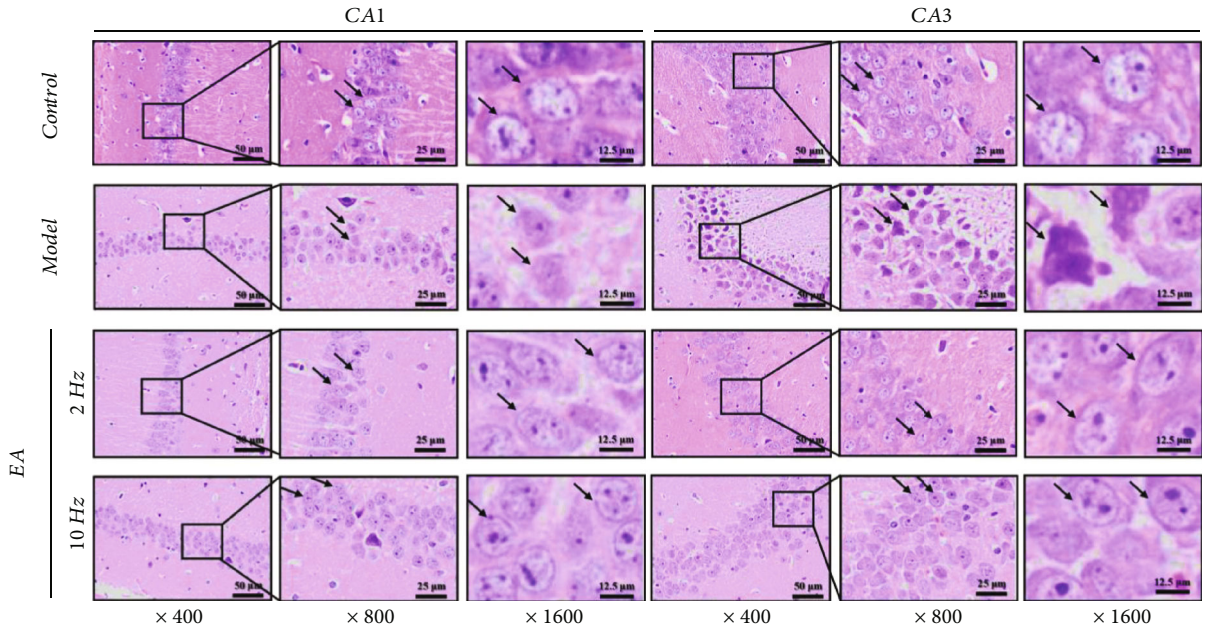


Figure 3: Electroacupuncture (EA) treatment was neuroprotective in the hippocampal CA1 and CA3 regions of SAMP8 mice. Magnification times, ×400, ×800, and ×1600. Bar, 50 μm, 25 μm, and 12.5 μm.

2 Hz, EA at 10 Hz can effectively reduce the number and ratio of positive cells in the CA1 region (Figure 4(b)) ($P < 0.05$).

3.4. EA Treatment at the GV20 and ST36 Acupoints Decreased Serum Inflammatory Factor Levels in SAMP8 Mice. We detected the levels of four common inflammatory factors, IL-1β, IL-6, IL-18, and TNF-α, in the serum of mice and found that compared with the control group the levels of four inflammatory factors in the model group were significantly increased ($P < 0.001$). After 14 days of EA treatment, the levels of four inflammatory factors decreased significantly ($P < 0.001$ or $P < 0.01$) (Figures 5(a)–5(d)). Compared with 2 Hz EA therapy, 10 Hz EA significantly reduced the levels of IL-1β and IL-6 ($P < 0.05$) (Figures 5(a) and 5(b)).

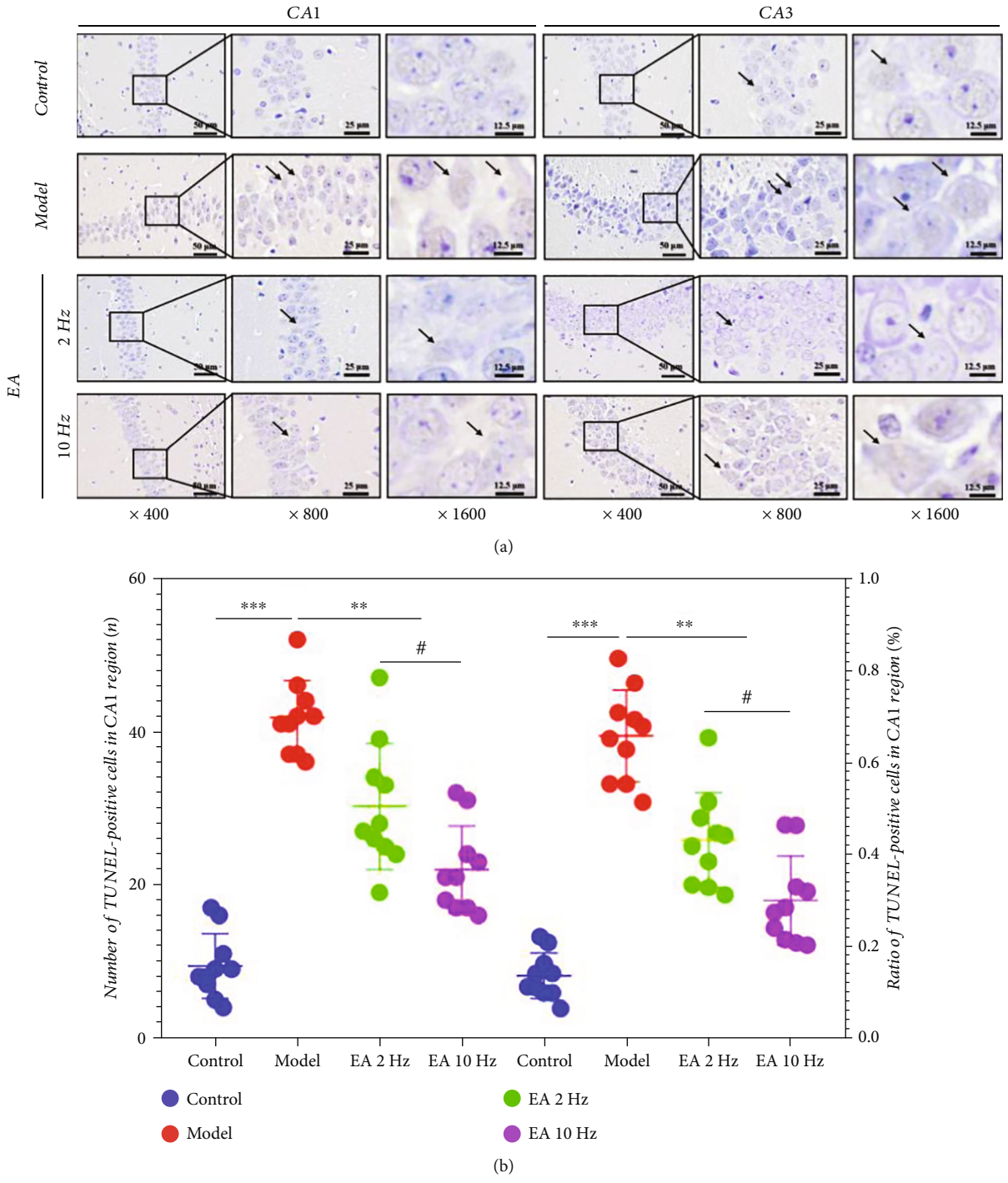
3.5. EA Treatment at the GV20 and ST36 Acupoints Acted via the NLRP3/Caspase-1 Pathway to Improve Cognitive Function in SAMP8 Mice. We measured the expression of 8 proteins, including NLRP3, ASC, caspase-1, GSDM-D, IL-1β, IL-18, Aβ, and tau, in the hippocampal tissues of mice and found that compared with the control group the model group had increased expression of 7 proteins (all but tau; $P < 0.001$ for each comparison). Compared with the model group, the expression of 5 proteins, including NLRP3, caspase-1, GSDM-D, IL-1β, and IL-18, was significantly decreased after 14 days of 2 Hz EA treatment ($P < 0.001$). After 14 days of 10 Hz EA treatment, the expression of 6 proteins, including NLRP3, ASC, caspase-1, GSDM-D, IL-1β, and IL-18, was significantly decreased compared with the model group ($P < 0.001$). Compared with the 2 Hz EA group, the expression of 5 proteins, NLRP3, ASC, caspase-1, IL-1β, and IL-18, in the 10 Hz group was significantly downregulated ($P < 0.05$ or $P < 0.01$) (Figures 6(a)–6(i)). Interestingly, EA (2 Hz and 10 Hz) treatment had no significant effect on the expression of Aβ and tau proteins in the hippocampal tissues of SAMP8 mice, and the difference was not significant ($P > 0.05$) (Figures 6(a) and 6(h)–6(i)).

4. Discussion

The purpose of our study was to investigate whether SAMP8 mice with CI would benefit from different frequencies of EA treatment commonly used in clinical and previous studies [19, 20] and to explore the underlying mechanism based on NLRP3/caspase-1-mediated hippocampal pyroptosis induced by the chronic inflammatory cascade. The findings clearly suggest that EA treatment does have the hypothesized effect.

4.1. EA Is an Effective Therapy for Cognitive Impairment Caused by Aging. In recent years, a growing amount of evidence has confirmed EA as an effective therapy for a wide variety of diseases featuring cognitive dysfunction, such as Alzheimer's disease (AD) [21], vascular dementia (VD) [22], and CI [23, 24]. For example, EA treatment at the GV20 and ST36 acupoints improves model animal learning and memory abilities and protects against hippocampal injury, inhibits inflammatory factors, and regulates brain activity via antioxidative damage [25, 26]. An increasing number of countries have endorsed the efficacy and safety of EA treatment [27, 28]. These considerations led us to further explore the potential improving cognitive effects of EA treatment on CI in our SAMP8 mouse model.

In this study, we found that EA treatment at the GV20 and ST36 acupoints improved cognitive function in SAMP8 mice. According to our previous studies and other reports in the literature [18, 29], 7-month-old SAMP8 mice showed



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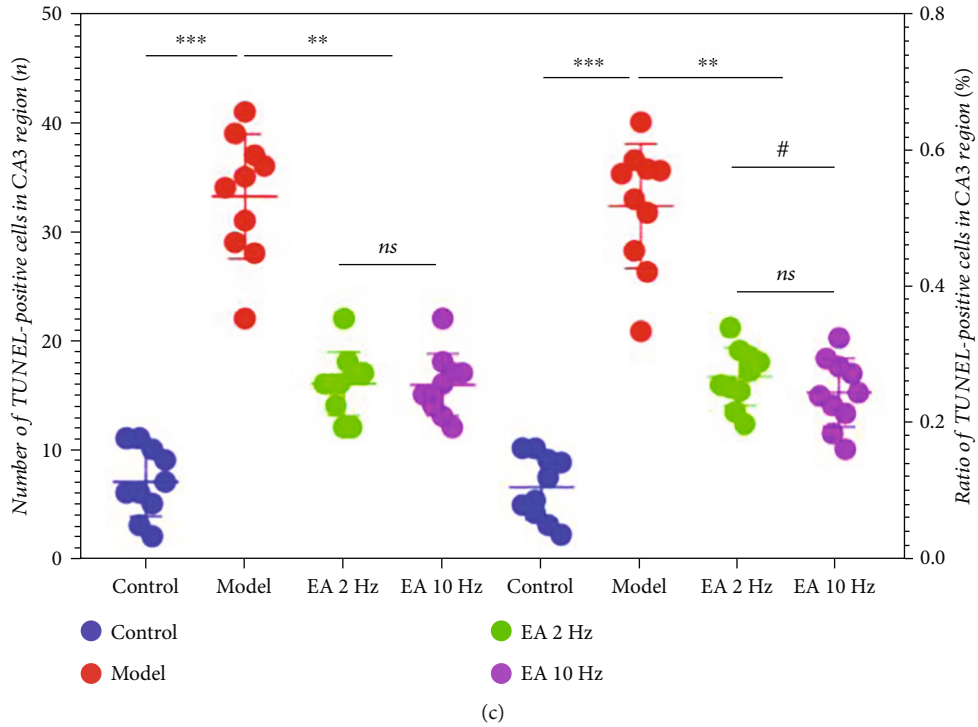

Figure 4: Electroacupuncture (EA) treatment limits neuronal cell death in the hippocampal CA1 and CA3 regions in SAMP8 mice. (a) Representative images of TUNEL staining in the hippocampal CA1 and CA3 regions from SAMR1 or SAMP8 mice. Magnification, ×400, ×800, and ×1600. Scale bars, 50 μm, 25 μm, and 12.5 μm. (b) The number and ratio of TUNEL-positive cells in the CA1 region. (c) The number and ratio of TUNEL-positive cells in the CA3 region. Data are the mean ± SD ($n = 10$ per group). $^{***}P < 0.001$ and $^{**}P < 0.01$ vs. the model group; $\#P < 0.05$ vs. the 2 Hz EA group. ns: not significant.

significant CI, and SAMR1 mice of the same age were selected as controls. Both animals are ideal models for studying CI induced by aging and are also ideal animal models for drug screening and treatment evaluation [30]. After 14 days of EA treatment, the learning ability of the animals in each group was investigated by navigation experiments for 5 consecutive days. EA at low (2 Hz) and high (10 Hz) frequencies effectively reduced the escape latency and travel distance of SAMP8 mice and resulted in clearer swimming tracks. Compared with the 2 Hz EA treatment, the 10 Hz EA treatment improved the learning ability of SAMP8 mice more significantly, but the difference was not significant ($P > 0.05$). On the sixth day, a spatial exploration experiment was conducted to investigate the memory ability of the animals in each group. EA at both frequencies effectively improved the crossing times of the target platform of SAMP8 mice, but no significant difference was found between the EA groups ($P > 0.05$). In the Morris water maze experiment, we did not observe significantly decreased swimming speed due to physical exhaustion and other factors during 6 consecutive days of testing, indicating that the Morris water maze is safe and reliable as a behavioral standard for evaluating the cognitive function of animals, and the experimental results will not be affected if each group is under the same experimental conditions. The above evidence suggests that EA treatment at either frequency can effectively improve the cognitive function of SAMP8 mice.

4.2. Increase in EA Frequency Plays a Better Role in Inhibiting Inflammation and Hippocampal Cell Death. The chronic inflammatory cascade reaction induced by aging is the main factor stimulating the death of hippocampal neurons. Our previous study found that IL-1β, IL-6, IL-18, and TNF-α were the inflammatory factors that were significantly increased in the serum of SAMP8 mice [18]. After 14 days of EA treatment, we found that both frequencies of EA treatment effectively reduced the four inflammatory factors. Compared with the 2 Hz EA treatment, the 10 Hz EA treatment has an advantage in reducing serum IL-1β and IL-6 levels. Cognitive function is critically related to the hippocampal CA1 and CA3 regions, which were observed in this study [18]. Compared with the hippocampus of SAMR1 mice, we found that those of SAMP8 mice exhibited a disordered pyramidal cell arrangement, incomplete membrane structure, shrinking nuclei, and other abnormal pathological findings, which was consistent with most previous reports using SAMP8 mice as model animals. More importantly, we found that EA, especially the 10 Hz EA treatment, can effectively improve these typical pathological changes, which are more obvious.

Hippocampal TUNEL staining and quantitative analysis are common detection methods conducted on the death of neurons. Compared with SAMR1 mice, SAMP8 mice were characterized by increased numbers and ratios of TUNEL-positive cells in the hippocampal CA1 and CA3 regions.

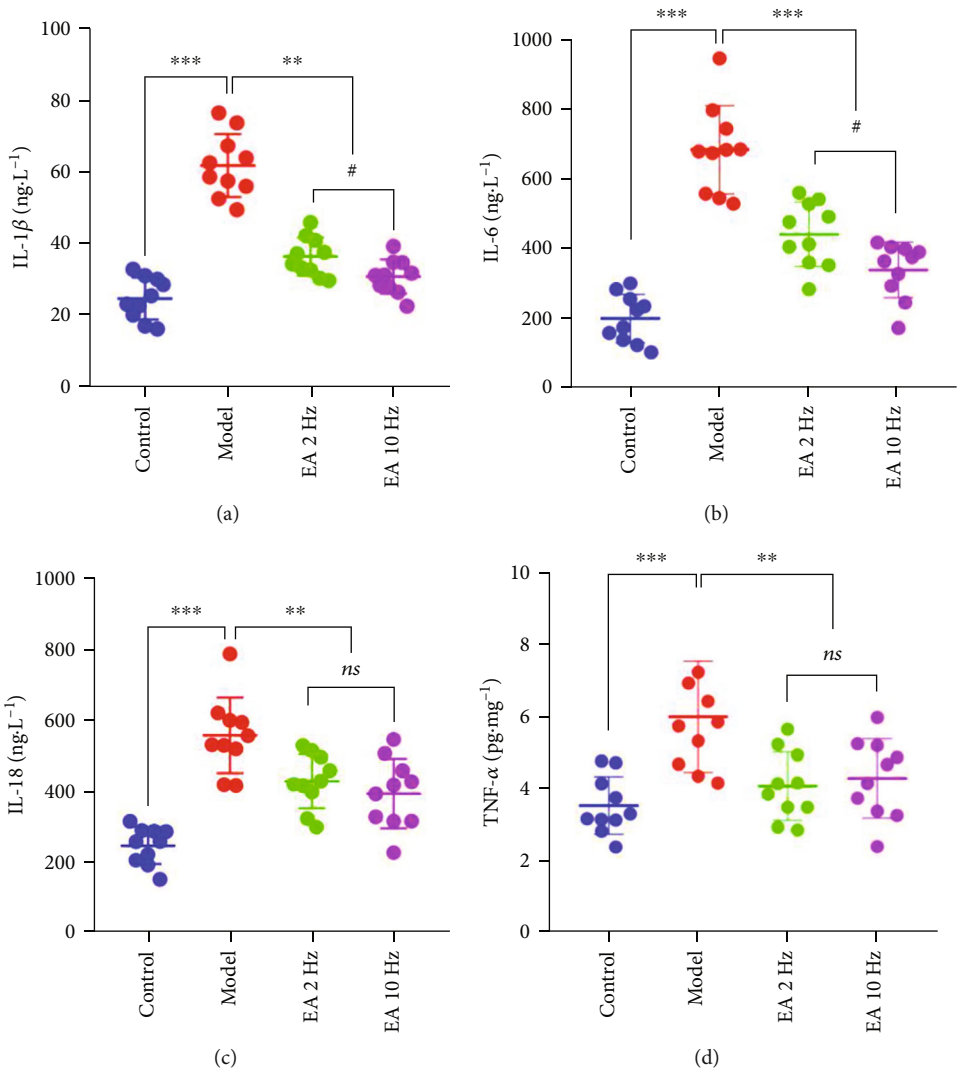

FIGURE 5: Electroacupuncture (EA) treatment attenuated the serum inflammatory factors in SAMP8 mice. (a) The level of interleukin-1β (IL-1β). (b) The level of interleukin-6 (IL-6). (c) The level of interleukin-18 (IL-18). (d) The level of tumor necrosis factor α (TNF-α). Data are presented as the means ± standard error of the mean ($n = 10$ per group). $^{***}P < 0.001$ and $^{**}P < 0.01$ vs. the model group; $\#P < 0.05$ vs. the 2 Hz EA group. ns: not significant.

The model mouse hippocampus may have neuronal cell death induced by inflammation, and this mode of cell death has been the focus area of CI in recent years. After 14 days of EA treatment, we found that EA could effectively reverse the cell death and the ratio of TUNEL-positive neurons in the hippocampal CA1 and CA3 regions and that 10 Hz EA treatment had a more significant reversal effect on the CA1 region than 2 Hz EA treatment. Furthermore, the increase in EA frequency may play a better role in inhibiting inflammation and hippocampal cell death.

4.3. High-Frequency EA Therapy Can Effectively Inhibit Hippocampal Pyroptosis through the NLRP3/Caspase-1 Pathway. At present, CI is believed to be mainly induced by abnormal changes in the tau protein framework, resulting in neurofibrillary tangles and the formation of senile plaques caused by excessive deposition of β-amyloid (Aβ) [31]. In addition, various in vivo and in vitro experiments have proven that pyroptosis is related to the pathogenesis of CI [5, 32], but the specific mechanism by which it participates remains unclear. The inflammatory response is a protective mechanism initiated by immune cells in response to injury- or infection-related factors; meanwhile, a long-term excessive inflammatory response may aggravate neural plasticity damage and disease progression. The neuroinflammatory response is generally believed to be regulated by NLRP3 inflammasome-dependent pyroptosis of neurons, and the death of neurons caused by pyroptosis is closely related to the onset of cognitive impairment [33].

The NLRP3 inflammasome comprises NLRP3, ASC, and caspase-1 [34]. GSDM-D (gasdermin-d) is the substrate of caspase-1 [35]. After GSDM-D protein is activated by caspase-1, it can cause cell membrane rupture, allowing water molecules and other substances to enter cells [36]; it can thereby induce the release of a large number of inflammatory cytokines, including IL-1β and IL-18, causing cell pyroptosis

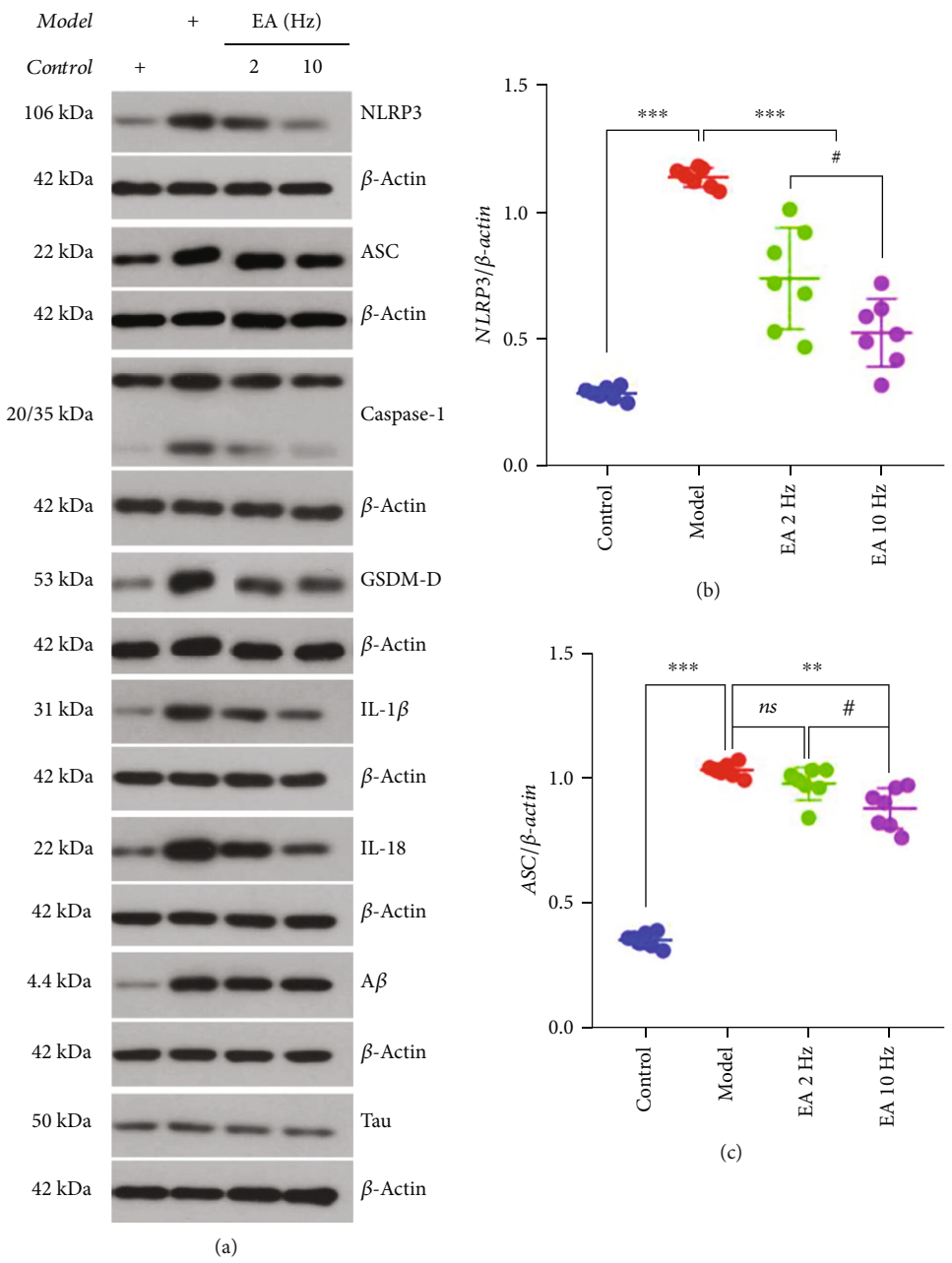

Figure 6: Continued.

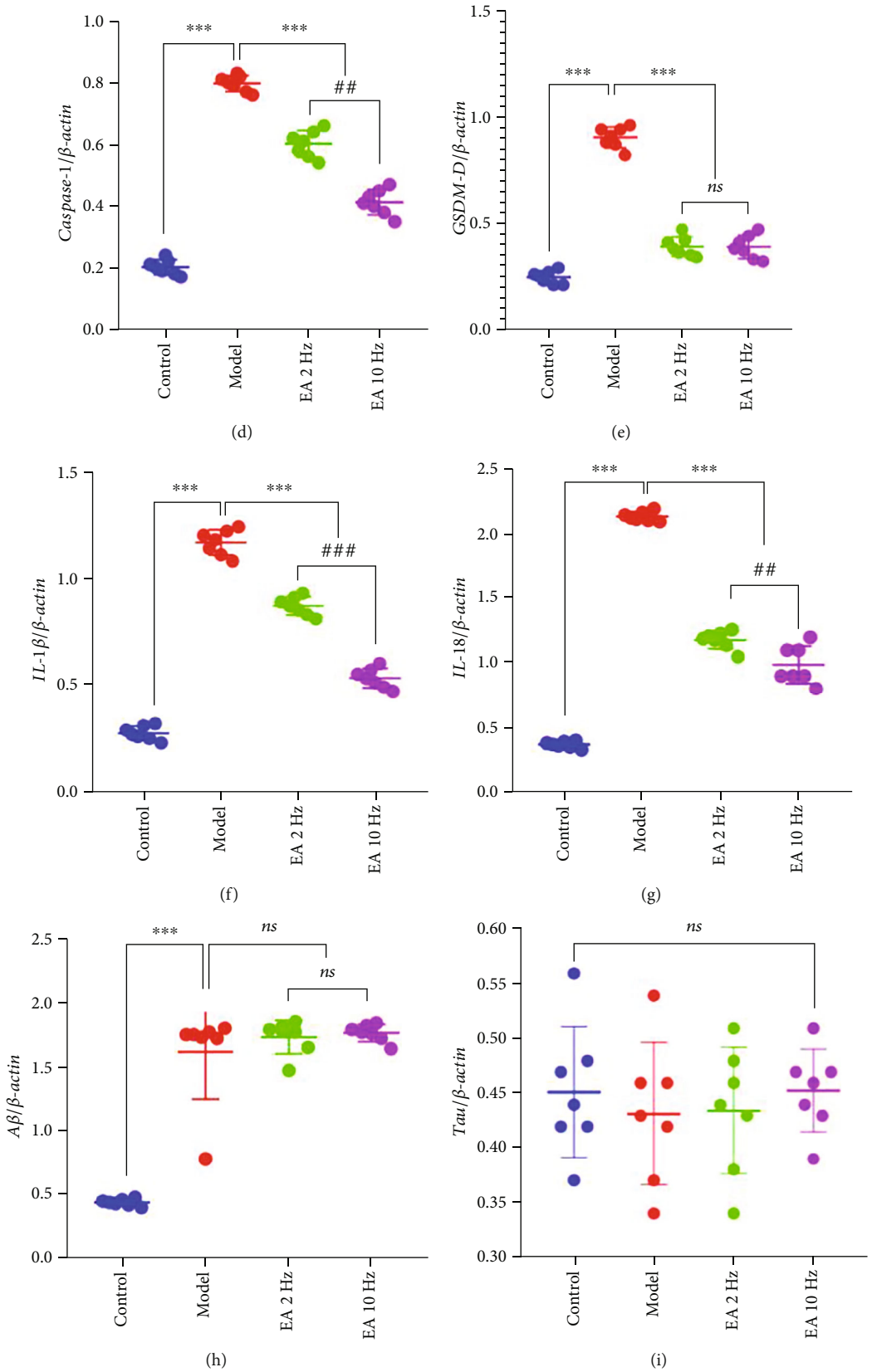

Figure 6: Expression of NLRP3/caspase-1 signaling-related proteins in the hippocampal tissue. (a) Western blot images showing the protein levels of NLRP3, ASC, caspase-1, GSDM-D, IL-1β, IL-18, Aβ, and tau in the hippocampus. (b–i) Quantification of NLRP3, ASC, caspase-1, GSDM-D, IL-1β, IL-18, Aβ, and tau bands in the hippocampus. Data are presented as the means ± standard error of the mean ($n = 7$ per group). $^{***}P < 0.001$ vs. the model group; $^{###}P < 0.001$ and $^{##}P < 0.01$ vs. the 2 Hz EA group. ns: not significant.

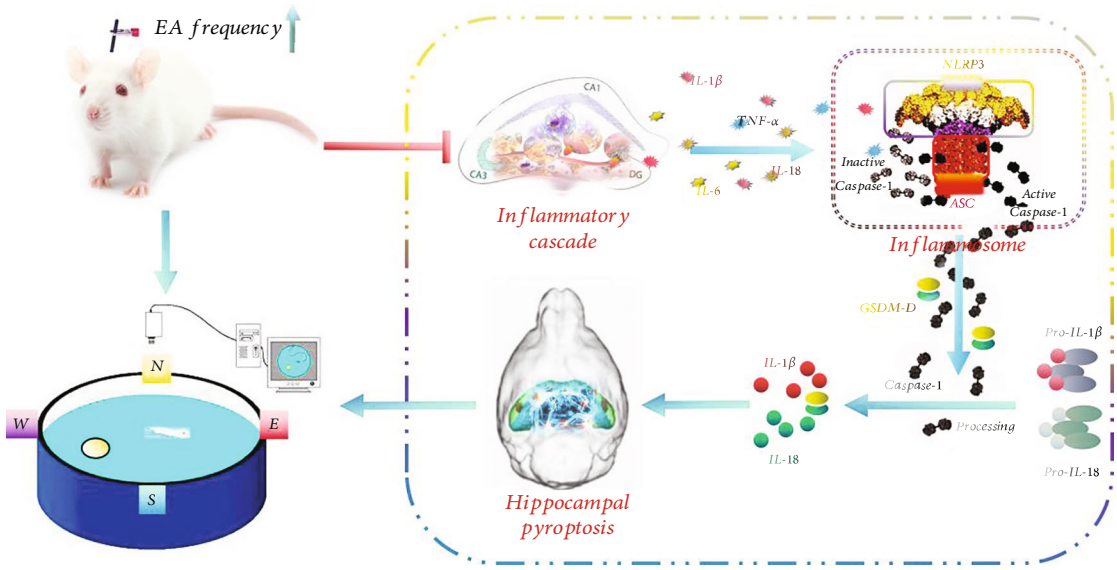

Figure 7: EA inhibited the activation of NLRP3 inflammasome to improve cognitive impairment. The attenuation of NLRP3 inflammasome activation ultimately reduced IL-1β, IL-18, and GSDM-D expressions and attenuated the inflammatory response of the hippocampus, thereby inhibiting hippocampal pyroptosis to improve cognitive impairment in SAMP8 mice.

[36, 37]. Studies have shown that the NLRP3 inflammasome can identify Aβ and thus play an important role in the process [38]. Activated soluble interleukin-1 receptor type II, IL-18, and caspase-1 protein in cerebrospinal fluid (CSF) with mild CI and AD patients has been found by Lindberg et al. [39], indicating that inflammasome activation may be an important step in the development of early CI. Knocking out NLRP3 and caspase-1 in mouse models can largely prevent mice from developing CI-related learning and memory ability impairment and a serious pathological state [40]. Interestingly, a recent study showed that the absence of NLRP3 protected mice from aging-related inflammation and CI even in the brains of mice without excessive Aβ deposition [41]. Therefore, the inflammatory response dependent on NLRP3 is closely related to the cognitive decline associated with Aβ excessive deposition.

We found that the expression of NLRP3/caspase-1 pathway-related 6 proteins and Aβ protein in the hippocampus was significantly increased in SAMP8 mice compared with SAMR1 mice. Interestingly, no significant difference was found in tau protein expression, consistent with previous studies showing that extensive deposition of Aβ protein was a typical pathological characteristic in the hippocampal tissues of SAMP8 mice. This evidence suggests that CI in SAMP8 mice is related to NLRP3/caspase-1 pathway-mediated pyroptosis and excessive deposition of Aβ protein. After 14 days of EA treatment, NLRP3/caspase-1 pathway-related proteins were significantly downregulated; the 2 Hz EA treatment did not effectively reduce the expression of ASC protein, a component of the NLRP3 inflammasome, but the 10 Hz EA treatment was effective. This suggests that higher EA frequencies are more effective in inhibiting NLRP3/caspase-1 pathway-mediated cell pyroptosis (Figure 7). In other words, increasing the EA frequency can effectively inhibit hippocampal pyroptosis under the condition of acupoint determination, and this finding has not been reported in the relevant literature yet. Our findings suggest that the frequency of EA therapy plays a crucial role in the treatment of CI, and the underlying mechanism of this phenomenon is that the inhibitory role of the inflammatory cascade-induced activation of NLRP3 inflammasome is enhanced with the increase in EA frequency within a reasonable range.

4.4. EA Could Not Reduce the Aβ and Tau Protein Expression of SAMP8 Mice and the Limitations of This Study. Interestingly, we found that the two common EA treatments failed to reduce the expression of Aβ and tau proteins in the hippocampal tissues of SAMP8 mice after 14 days. Due to the lack of high-quality reports on the mechanism of EA treatment on cognitive function in SAMP8 mice, analyzing the underlying causes is difficult. Notably, a recent report using EA treatment at the KI3 acupoint to intervene in 5XFAD mice found that EA can downregulate the expression level of Aβ protein [21]. We analyzed whether the failure to reduce the expression level of Aβ in this study may be related to acupoint selection. The reason why no significant change in tau protein was found may be related to the absence of such pathological characteristics in the SAMP8 model mice.

The limitations of this study are as follows. First, acupoint specificity is the main factor influencing acupuncture outcomes in clinical and animal studies. Whether EA at the GV20 and ST36 acupoints is the best choice or whether EA at other acupoints improves cognitive function in SAMP8 mice with CI through the restoration of the NLRP3/caspase-1 signaling pathway needs to be further explored. Second, acupuncture has bidirectional regulatory effects under different functional conditions; thus, a follow-up is necessary for conducting EA research on the changes in mouse electroencephalogram (EEG). Third, previous studies have found that pyroptosis occurs faster than other forms of cell death,

such as apoptosis. The acupoint ST36 is on the leg, and the GV 20 acupoint is on the head. How the exact pathways that transduce the EA signal at two relatively distant acupoints to the hippocampus to affect protein translation in the animal model needs further exploration.

5. Conclusion

In conclusion, the present study provides evidence that EA treatment improves cognitive function, reduces inflammation, and inhibits pyroptosis in SAMP8 mice. The inhibition of NLRP3/caspase-1 signaling in the hippocampus may be involved in the beneficial effect of EA treatment on cognitive function.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that no conflicts of interest exist regarding the publication of this paper.

Authors' Contributions

Zhongren Sun, Jing Chen, and Hongcai Shang designed the experiments. Zhitao Hou, Meng Wang, Tingting Mei, Yue Zhang, Liying Song, and Xianming Shao performed the experiments. Ruijin Qiu, Qingshuang Wei, and Yitian Liu analyzed the data and revised the manuscript. All authors discussed the results and commented on the manuscript. All authors approved the final version of manuscript for submission. Zhitao Hou and Ruijin Qiu contributed equally to this work.

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Review Article

Evaluation and Treatment of Vascular Cognitive Impairment by Transcranial Magnetic Stimulation

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The exact relationship between cognitive functioning, cortical excitability, and synaptic plasticity in dementia is not completely understood. Vascular cognitive impairment (VCI) is deemed to be the most common cognitive disorder in the elderly since it encompasses any degree of vascular-based cognitive decline. In different cognitive disorders, including VCI, transcranial magnetic stimulation (TMS) can be exploited as a noninvasive tool able to evaluate *in vivo* the cortical excitability, the propension to undergo neural plastic phenomena, and the underlying transmission pathways. Overall, TMS in VCI revealed enhanced cortical excitability and synaptic plasticity that seem to correlate with the disease process and progression. In some patients, such plasticity may be considered as an adaptive response to disease progression, thus allowing the preservation of motor programming and execution. Recent findings also point out the possibility to employ TMS to predict cognitive deterioration in the so-called "brains at risk" for dementia, which may be those patients who benefit more of disease-modifying drugs and rehabilitative or neuromodulatory approaches, such as those based on repetitive TMS (rTMS). Finally, TMS can be exploited to select the responders to specific drugs in the attempt to maximize the response and to restore maladaptive plasticity. While no single TMS index owns enough specificity, a panel of TMS-derived measures can support VCI diagnosis and identify early markers of progression into dementia. This work reviews all TMS and rTMS studies on VCI. The aim is to evaluate how cortical excitability, plasticity, and connectivity interact in the pathophysiology of the impairment and to provide a translational perspective towards novel treatments of these patients. Current pitfalls and limitations of both studies and techniques are also discussed, together with possible solutions and future research agenda.

1. Introduction

1.1. Vascular Cognitive Impairment. Globally, vascular cognitive impairment (VCI) is defined as a decline in cognition due to cerebrovascular injury. It is currently viewed as an "umbrella term" encompassing mild VCI, vascular dementia (VaD), and mixed dementia [1–3]. Mild VCI is a decline in cognition not fully satisfying the diagnostic criteria for dementia [4, 5]. VaD identifies cognitively impaired patients who have lost their functional independence due to vascular lesions and includes different subtypes, such as poststroke dementia, multi-infarct dementia, strategic infarct dementia,

and the subcortical ischemic VaD. Finally, mixed dementia is the result of both vascular and degenerative pathophysiology, most commonly of Alzheimer's disease- (AD-) type [6]. Hence, VCI is deemed to be the most common cognitive disorder in the elderly, with a growing impact on patients' quality of life (QoL) and on social and healthcare system [2]. Moreover, vascular-derived impairment has a great prevalence in all types of cognitive decline, where its contribution to the deficits is considerable. Of note, this is the only contribution that can be, at least in part, treatable and preventable [7, 8].

In addition to the affected cognitive domains, which typically are attention, processing speed, and executive functioning [9], VCI can impact also on several neuropsychiatric aspects, such as behavioral and mood disturbances, making this disorder extremely heterogeneous [10–12]. Apathy, irritability, disinhibition, and psychomotor retardation are common examples of the behavioral changes found in VCI patients, while depression is the most reported mood disorder. Behavioral and mood changes correlate with the worsening of cognitive and functional status and significantly reduce the QoL of patients and caregivers [13–15].

The pathophysiology accounting for cognitive and behavioral-mood dysfunction in VCI is still not completely defined. The so-called "disconnection hypothesis," based on the analysis of brain images of large samples [16, 17], points to the result of a "disruption" of cortical and/or subcortical loops implicated in cognition and mood-affect regulation, due to acute of chronic cerebrovascular lesions [18–20]. In magnetic resonance imaging (MRI) of stroke or cerebral small vessel disease, ischemic white matter lesions (WMLs) are clinically associated with cognitive impairment [21]. In large community-based populations [16, 22, 23], WMLs are also associated with nonmotor sequelae, and cognitive and mood-behavior impairment was especially linked with the ischemic disruption of the prefrontal cortical-subcortical circuits [24]. In stroke survivors, the atrophy of the medial temporal lobe predicts early cognitive dysfunction [25]. Even subcortical ischemic vascular disease, including silent lacunar infarcts and WMLs, is associated with executive dysfunction and late-life depression, which is a clinical and neuroimaging condition now referred as "vascular depression" [20]. Taken together, it has been clearly established that cognitive limitations and depressive disorders are tightly intertwined in patients with both acute and chronic cerebrovascular diseases, such as stroke and small vessel disease, respectively [9, 11, 13, 26].

VCI diagnosis must capitalize from clinical and neuropsychological evaluations, as well as from structural and functional neuroimaging [11]. However, the search for novel hallmarks of disease process and progression, such as serological, cerebrospinal fluid (CSF), and instrumental markers, is needed to allow an early, tailored, and accurate screening of VCI patients. This will also pave the way to innovative therapeutic strategies and to the identification of predictors of drug response [27, 28]. Moreover, the noninvasively investigation of cortical circuitry in VCI patients has produced intriguing findings on abnormal cortical connectivity [29] and plasticity [30, 31].

Overall, neural plasticity refers to the brain's ability, particularly of the cerebral cortex, of reorganizing and adapting to different constantly changing environmental stimuli. This takes place through phenomena of modification of synaptic connection strength (like long-term potentiation (LTP) and long-term depression (LTD)), modification of the representation pattern and neuronal activity, modulation of gene induction and expression, changes in cerebral blood flow, and neurotrophin release [32]. Neural plasticity is an essential substrate for learning and memory [33], and its involvement in dementia (such as AD), movement disorders (such as Parkinson's disease), and neuropsychiatric disorders (such as major depression) [34] is well documented. Although abnormalities in brain plasticity and its components have been widely demonstrated in dementia, their role in the pathophysiology of VCI and in the counteraction against disease progression is still not understood. In this scenario, the contribution of noninvasive and translational brain stimulation techniques, namely, transcranial magnetic stimulation (TMS), is becoming of pivotal importance.

1.2. Transcranial Magnetic Stimulation. From the pioneering application of TMS to assess the primary motor cortex (M1) and the cortical-spinal conductivity [35], scientists boost the potentialities of this technique, which is employed today to study cortical excitability, to map connectivity, and to probe the propensity to undergo plastic phenomena [36]. This gives novel insights into the pathophysiology underlying several neurological and neuropsychiatric diseases [37, 38].

A functional assessment of global cortical excitability and cortico-spinal conduction results from the application of single magnetic pulses at adequate stimulator intensity over the M1 that elicits a motor evoked potential (MEP) recordable on the contralateral target muscle [39, 40]. The MEP amplitude is an aggregate measure of the excitation state of M1's output cells, nerve roots, and the conduction along the peripheral motor nerves till the muscles [41]. The resting motor threshold (rMT), i.e., the minimum intensity of stimulation needed to evoke a MEP, is a basic index of M1 excitability, as it is a compound measure of the membrane excitability of cortical neurons, the neural inputs into pyramidal cells within the cortex, and the excitability of spinal motor neurons, neuromuscular junctions, and muscles [42].

During a tonic contraction of the contralateral muscles, the result of a suprathreshold TMS pulse applied to the M1 is a few hundred milliseconds suppression of the electromyographic (EMG) activity of those muscles [43]. This phenomenon, called contralateral cortical silent period (cSP), is exploited as a functional measure of intracortical inhibitory circuits [44, 45], mainly mediated by the gamma-aminobutyric acid- (GABA-) B transmission [46]. Conversely, activation of the muscle and stimulation of the hemisphere of the same side evoke the ipsilateral silent period (iSP), which it is thought to receive some modulated effects from transcallosal output neurons that project to contralateral GABAergic interneurons [47, 48].

Paired-pulse TMS paradigm allows the assessment of the short-interval intracortical inhibition (SICI) and the intracortical facilitation (ICF) of the motor response [49, 50].

The activity of GABA-A interneurons is the most likely mediator of SICI [51, 52], whereas the neurophysiology of ICF is more complex. It probably relates to the activation of a cortical circuit projecting upon cortico-spinal cells different from that preferentially activated by single-pulse TMS. ICF seems dependent to a great extent on the activity of glutamatergic excitatory interneurons, although other mediators are known to contribute [53, 54].

Researchers have also the possibility of investigating the sensory-motor interactions in the cerebral cortex by using specific TMS protocols. One of these allows for the investigation of the short-latency afferent inhibition (SAI), which mainly reflects the central cholinergic circuits' integrity [55]. Indeed, while the muscarinic antagonistic scopolamine in healthy subjects reduces or abolishes SAI [56], acetylcholine positively modulates it [57]. It has been suggested that SAI may depend on the integrity of circuits linking sensory input and motor output [58, 59], thus providing valuable diagnostic information in a variety of cognitive and movement disorders [60, 61]. Finally, TMS also allows the study of synaptic plasticity through different paradigms of paired-associative stimulation (PAS), e.g., by applying a magnetic stimulus after a brief period of exercise or by using repetitive low-frequency median nerve stimulation combined with TMS over the contralateral M1 [62]. PAS has shown to lead to LTP-/LTD-like changes within the sensory-motor pathways [63].

Figure 1 schematically illustrates the technical aspects and the neurophysiological correlate of SICI, ICF, SAI, PAS, and repetitive TMS (rTMS).

1.3. Repetitive TMS. Repetitive TMS (rTMS) over the same cortical target induces a transient modification of the cortex excitability, which decreases by using low frequencies (≤1 Hz) and increases by using high frequencies (5-20 Hz) [64]. The neurobiology of rTMS seems to share many features with LTD and LTP's induction by tetanic stimulation in cortical slices [65], such as the dependence from N-methyl-D-aspartate- (NMDA-) receptor activity [66], the sensibility to prior synaptic activation [67], and the strict link with stimulation frequency [68]. The short-term changes in synaptic efficacy and the rapid downregulation of GABA-related inhibitory circuits are key processes of calcium- and sodium channel-dependent LTP plasticity [69, 70]. Conversely, by inducing LTD-like responses, rTMS decreases the synaptic efficacy [71, 72].

The effects of repeated sessions of rTMS persist in time and act by enhancing plasticity when needed but also by downregulating it when plasticity becomes inappropriate or even maladaptive [73]. For all those reasons, the translational therapeutic and rehabilitative applications of rTMS may cover a wide range of neurological and psychiatric disorders [74, 75]. Accordingly, in October 2008, the Food and Drug Administration (FDA) approved rTMS as an add-on treatment of adult drug-resistant major depressive disorder (MDD). Besides, specific rTMS paradigms, like the theta-burst stimulation [76] or the quadripulse stimulation [77], may help in a better comprehension of synaptic plasticity phenomena or even more complex responses, such as the metaplasticity (i.e., "plasticity of synaptic plasticity") [78–80].

Overall, rTMS is safe and well tolerated. A discomfort caused by scalp or facial muscle twitching and transient headache are the most commonly reported side effects [81], while the induction of seizures is a very rare but serious adverse effect, although not common even employing supratherapeutic stimulations [82]. Nevertheless, epileptic patients or those with risk factors of epilepsy should be managed with extreme caution.

1.4. Aim e Rationale. To date, the exact relationship between cognitive functioning, motor cortical excitability, and synaptic plasticity in VCI is not completely unveiled. In this work, we review all the TMS and rTMS studies related to VCI to provide a timely translational perspective on how cortical excitability and network connectivity interact to determine the pathophysiology and plastic changes in VCI and its subtypes, and how these findings may be exploited by experimental treatments. Current pitfalls and limitations of both studies and techniques are also discussed, together with possible solutions and future research agenda.

2. Methods

A literature search was carried out to find all the relevant studies of TMS and rTMS in VCI. A PubMed-based literature review was performed by using the following search queries:

(i) ("transcranial magnetic stimulation" [MeSH Terms] OR ("transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "transcranial magnetic stimulation" [All Fields] OR ("repetitive" [All Fields] AND "transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "repetitive transcranial magnetic stimulation" [All Fields]) AND "vascular" [All Fields] AND ("cognitive dysfunction" [MeSH Terms] OR ("cognitive" [All Fields] AND "dysfunction" [All Fields]) OR "cognitive dysfunction" [All Fields] OR ("cognitive" [All Fields] AND "impairment" [All Fields]) OR "cognitive impairment" [All Fields])

(ii) ("transcranial magnetic stimulation" [MeSH Terms] OR ("transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "transcranial magnetic stimulation" [All Fields] OR ("repetitive" [All Fields] AND "transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "repetitive transcranial magnetic stimulation" [All Fields]) AND ("dementia, vascular" [MeSH Terms] OR ("dementia" [All Fields] AND "vascular" [All Fields]) OR "vascular dementia" [All Fields])

(iii) ("transcranial magnetic stimulation" [MeSH Terms] OR ("transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "transcranial magnetic stimulation" [All Fields] OR ("repetitive" [All Fields] AND "transcranial" [All

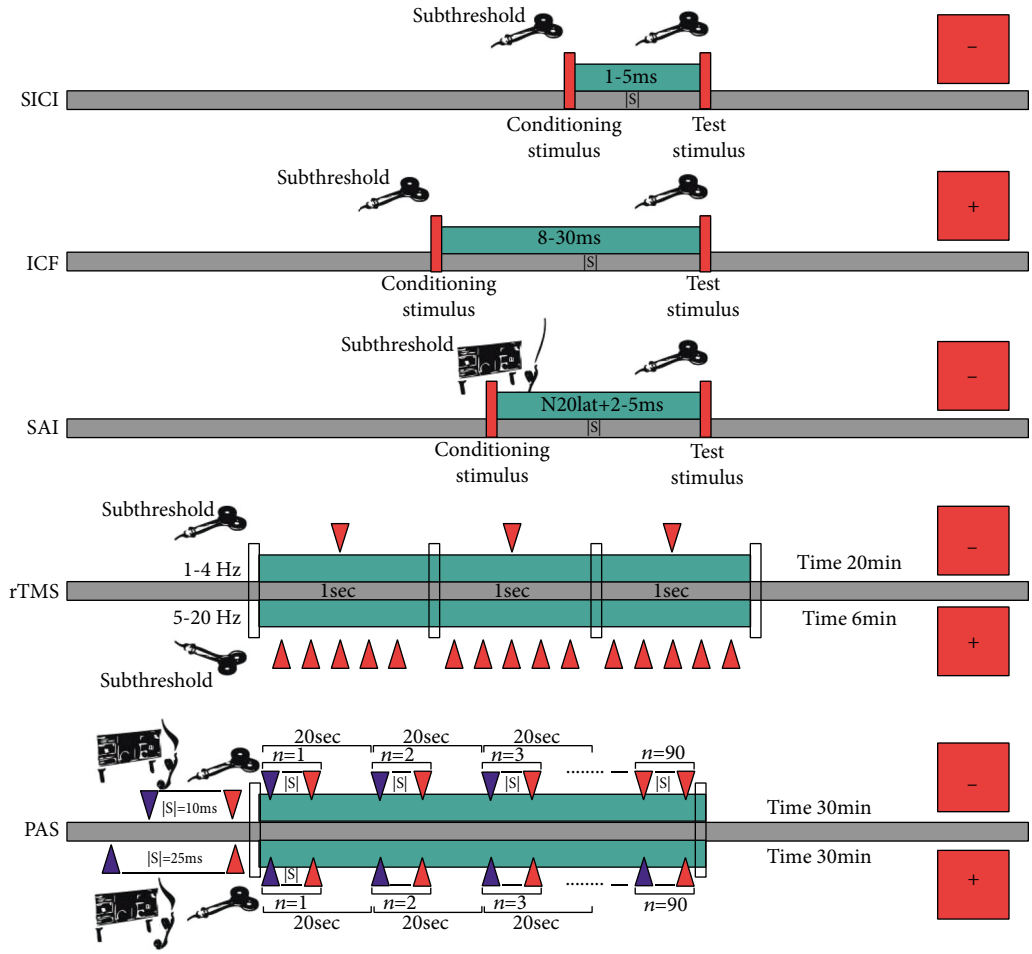

Figure 1: Schematic representation of some TMS measures and protocols of stimulation. Legend (in alphabetic order): ICF: intracortical facilitation; ISI: interstimulus interval; SAI: short-latency afferent inhibition; PAS: paired-associative stimulation; rTMS: repetitive transcranial magnetic stimulation; SICI: short-interval intracortical inhibition; +: facilitatory/excitatory effect; -: inhibitory/suppressive effect.

Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "repetitive transcranial magnetic stimulation" [All Fields]) AND "vascular" [All Fields] AND ("depressive disorder" [MeSH Terms] OR ("depressive" [All Fields] AND "disorder" [All Fields]) OR "depressive disorder" [All Fields] OR "depression" [All Fields] OR "depression" [MeSH Terms])

(iv) ("transcranial magnetic stimulation" [MeSH Terms] OR ("transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "transcranial magnetic stimulation" [All Fields] OR ("repetitive" [All Fields] AND "transcranial" [All Fields] AND "magnetic" [All Fields] AND "stimulation" [All Fields]) OR "repetitive transcranial magnetic stimulation" [All Fields]) AND ("cadasil" [MeSH Terms] OR "cadasil" [All Fields])

Two independent authors (FF and MP) screened titles and abstracts of all retrieved publications, and disagreements were solved by the consensus of a third author (RB). Duplicated entries, retracted publications, studies on other diseases different from VCI or its subtypes, works on animals or *in vitro*, studies without statistical analysis, non-English written papers, publications that are not research studies (i.e., commentaries, letters, editorials, and reviews), and any other article that did not fit with the scope of this review were excluded. Articles listed in the references were also reviewed in search of more data. We considered studies indexed from the database inception to April 2020.

3. Results and Discussion

A total of 77 results were originally retrieved. Of these, 20 peer-reviewed publications were selected according to the above inclusion and exclusion criteria. The examination of the references from relevant papers detected 5 additional studies fitting the purpose of this review. Eventually, a total of 25 papers were included (Figure 2), and their main findings were analyzed clustering within two groups, one on TMS studies (summarized in Table 1) and the other on rTMS studies (summarized in Table 2). More in details, we included in the TMS group 6 studies on mild VCI [30, 31, 83–86], 6 on VaD [87–92], 3 on vascular depression [93–95], and 4 on

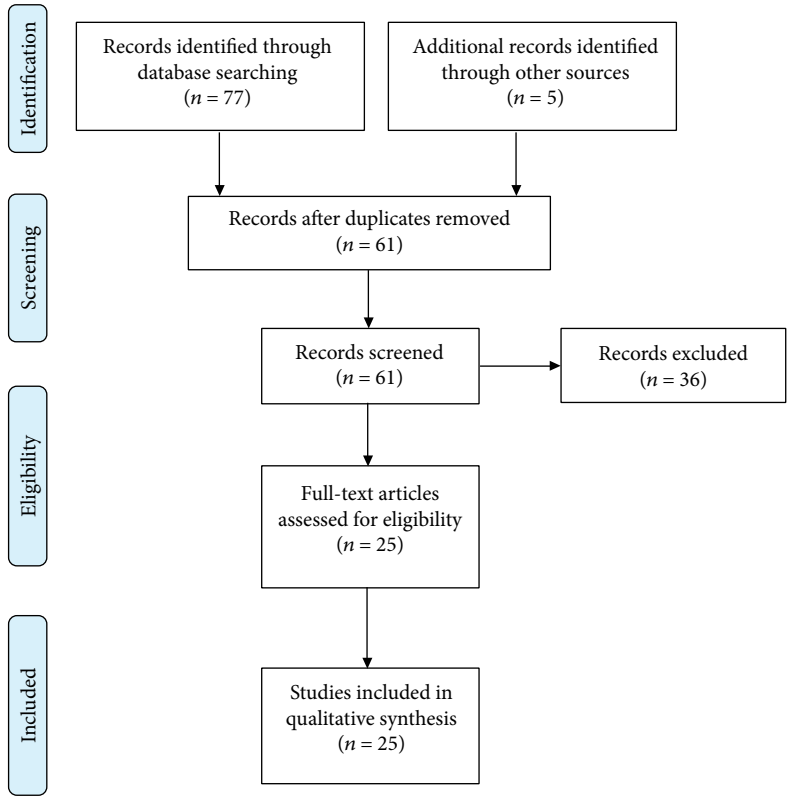

Figure 2: Flow diagram showing the search strategy, the number of records identified, and the number of included/excluded studies [106]. This figure is reproduced from Moher, David et al. preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. BMJ 2009; 339:b2535 (under the Creative Commons Attribution License/public domain).

cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [96–99], while the rTMS studies group consisted of 2 articles in mild VCI [100, 101] and 4 in vascular depression [102–105].

3.1. Mild Vascular Cognitive Impairment. The identification of mild VCI subjects at risk for clinical progression into VaD or mixed dementia is a crucial challenge for both clinicians and researchers because it may raise the chances to early diagnose and to delay the disease progression.

A previous study on nondemented elderly patients with subcortical ischemic vascular disease and clinical-cognitive profile of mild VCI [30] found that prefrontal subcortical loops lesioned by the ischemic interruption due to WMLs or lacunar infarcts lead to functional changes of the intracortical excitatory neuronal circuits (i.e., increased ICF). In this patient class, a further study has also shown that iSP is spared [83], unlike neurodegenerative disorders, such as AD and mild cognitive impairment (MCI), that show abnormal iSP since the early stages. This suggests a functional integrity of the transcallosal inhibitory connections in VCI, at least in the early phase [107].

A TMS study carried out on the same participants after a 2-year follow-up [31] found that, compared to the baseline, patients exhibited an increased global cortical excitability (reduction of the median rMT) and a significant worsening of the score of neuropsychological tests evaluating the frontal lobe abilities. The researchers interpreted the findings as indicative of plastic compensatory mechanisms in response to cortical disconnection [31, 108]. In particular, the study hypothesizes that rMT might become abnormal when VCI progresses to VaD and that its value can be used as a "neurophysiological cut-off" segregating patients who will progress from those who will remain cognitively stable. It is known, indeed, that higher motor cortex facilitation marks higher risk to convert from normal aging brain to cognitive impairment up to an overt dementia [31]. These findings are in line with the observation of enhanced cortical plasticity and reorganization, probably as compensatory mechanisms due to impaired cerebral autoregulation, in nondemented patients with severe ischemic small vessel disease [84, 85].

Notably, mild VCI individuals do not show impaired cholinergic activity compared to age-matched controls [86], which might suggest a distinctive cholinergic profile characterizing the early stages of VaD and differentiating it from the "cholinergic" forms of cognitive decline, such as MCI and AD [109]. However, cholinergic involvement in VaD is still under debate, and the few available TMS data show conflicting results [88, 89, 91]. The high heterogeneity in the location and severity of subcortical infarcts, leading to variations in the resultant distribution and magnitude of the cholinergic denervation, may be a reasonable explanation [91]. Finally, SAI might be useful in the identification of responders to the acetylcholinesterase inhibitors and, indirectly, in the differentiation between "cholinergic" and "non-cholinergic" forms of dementia [86].

Table 1: TMS studies in patients with vascular cognitive impairment.

VCI subtype	Study, year	Study design	Patients *n*	Main findings
Mild VCI	Bella et al., 2011 [30]	Cross-sectional	10	↑ Intracortical excitatory neuronal circuits
	Bella et al., 2013 [31]	Case-control	9	↑ Excitability during the progression of VCI
	Lanza et al., 2013 [83]	Cross-sectional	15	= Transcallosal inhibitory functioning, unlike AD and mild cognitive impairment
	List et al., 2013 [84]	Cross-sectional	20	↑ Cortical plasticity as a compensatory mechanism
	List et al., 2014 [85]	Cross-sectional	12	↓ LTP-like plasticity in the affected hemisphere = Motor learning between hemispheres, maybe due to a GABA-B effect in the affected side
	Bella et al., 2016 [86]	Cross-sectional	25	Central cholinergic pathway not clearly affected
Vascular dementia	Alagona et al., 2004 [87]	Cross-sectional	20 AD 20 SIVD 20 HC	↓ Motor threshold in SIVD compared to AD and HC
	Di Lazzaro et al., 2008 [88]	Cross-sectional	12 VaD 12 AD 12 HC	= Short-latency afferent inhibition in VaD patients and significantly reduced in AD
	Nardone et al., 2008 [89]	Cross-sectional	20 SIVD 25 HC	↓ Mean short-latency afferent inhibition in patients
	Pennisi et al., 2011 [90]	Cross-sectional	20 VaD 20 mild VCI	↑ Cortical excitability in demented patients only
	Nardone et al., 2011 [91]	Cross-sectional	28	Microbleeds on cholinergic function are independent of white matter lesion extent and ischemic stroke
	Guerra et al., 2015 [92]	Cross-sectional	7 VCI 9 AD	↑ Excitability and plasticity in AD and VaD Hyperexcitability promoted plasticity
Vascular depression	Bella et al., 2011 [93]	Cross-sectional	15 MDD 10 non-depressed	Neurophysiology of vascular depression differs from MDD, and it is similar to that of subcortical ischemic vascular disease
	Concerto et al., 2013 [94]	Cross-sectional	11 depressed 11 MDD	Different patterns of cortical excitability between late-onset vascular depression and early-onset nonvascular MDD
	Pennisi et al., 2016 [95]	Case-control	16 MDD 11 nondepressed	↑ Risk of dementia in vascular depression, probably due to subcortical vascular burden or to the lack of compensatory functional cortical changes
CADASIL	Manganelli et al., 2008 [96]	Cross-sectional	10 CADASIL 10 HC	↓ Short-latency afferent inhibition in patients ↓ Resting motor threshold significantly reduced in patients
	List et al., 2011 [97]	Cross-sectional	12 CADASIL 10 HC	↑ Cortical plasticity in patients compared to HC
	Palomar et al., 2013 [98]	Cross-sectional	10	Acetylcholine and glutamate were involved Abnormal sensory-motor plasticity correlated with cognition
	Nardone et al., 2014 [99]	Cross-sectional	8 CADASIL 8 AD	↓ Cholinergic functioning, with restoration by L-3,4-dihydroxyphenylalanine in AD group only

Legend (in alphabetical order): AD: Alzheimer's disease; CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; GABA: gamma-aminobutyric acid; HC: healthy controls; LTP: long-term potentiation; MDD: major depressive disorder; *n*: patients' number; SIVD: subcortical ischemic vascular disease; VaD: vascular dementia; VCI: vascular cognitive impairment; ↑: increase/enhancement; ↓: decrease/reduction; =: no significant change/modification.

3.2. Vascular Dementia. A common feature of AD and VaD patients is the increase of M1 excitability, (i.e., reduction of rMT), which differentiate them from normal brain aging [87]. Different studies converge on the hypothesis that an enhanced excitability and plasticity seems to have a role in counteracting cognitive decline in the elderly [110] as a compensatory response to neuronal loss and vascular injury [111]. However, this likely hypothesis warrants future experimental investigations on longitudinal studies and further clinical-pathological correlations. In AD patients, a reorganization of cortical functions has been reported since the early stages, likely due to the occurrence of a frontal and medial shift of the "center of gravity" of the TMS-based cortical motor map representations [112, 113]. A similar pattern has been shown also in subcortical ischemic VaD, which identifies a homogenous subtype of patients characterized by insidious onset, gradual course, and relatively slow progression, which make them hard to differentiate them from AD patients [114].

Although much less is known, plastic phenomena have been also reported to take place also in VaD. While exploring

Table 2: Repetitive TMS studies in patients with vascular cognitive impairment.

VCI subtype	Study, year	Study characteristics	Main findings
Mild VCI	Rektorova et al., 2005 [100]	Type of study: randomized, controlled, blinded, crossover Subjects: 7 Type of coil: figure-of-eight coil (7 cm diameter) Stimulation site: left DLPFC (active), left M1 (control) Stimulation frequency: 10 Hz Intensity: 100% rMT Length: 3 rTMS blocks, separated by a 10 min interval; in each block, fifteen 10-pulse trains, each of 1 s duration, delivered with an intertrain interval of 10 s Duration: 1 session Total number of pulses delivered: 450	Significant positive effect of active stimulation on the Stroop color-word interference test
	Sedlackova et al., 2008 [101]	Type of study: randomized, controlled, blinded, crossover Subjects: 7 Type of coil: figure-of-eight coil (7 cm diameter) Stimulation site: left DLPFC (active), left M1 (control) Stimulation frequency: 1 Hz; 10 Hz Intensity: 100% rMT Length: for the 10 Hz stimulation: 3 rTMS blocks, separated by a 10-minute interval; in each block, fifteen 10-pulse trains, each of 1 s duration, delivered with an intertrain interval of 10 s; for the 1 Hz stimulation: continuous Duration: 4 sessions (two at 1 Hz and two at 10 Hz) Total number of pulses delivered: 450 at 10 Hz; 1,800 at 1 Hz	Significant improvement in the Stroop color-word interference test after the stimulation of DLPFC but not M1; improvement in the digit symbol subtest of the Wechsler Adult Intelligence Scale-revised after rTMS, regardless of the stimulation site. No measurable effect in any other neuropsychological test
Vascular depression	Fabre et al., 2004 [102]	Type of study: open trial Subjects: 11 Type of coil: figure-of-eight coil Stimulation site: left prefrontal cortex Stimulation frequency: 10 Hz Intensity: 100% rMT Length: twenty 8 s trains, with 52 s intertrain intervals Duration: 10 sessions over two weeks	Five out of 11 patients responded to rTMS in terms of clinically meaningful improvement in HDRS scores, with a decrease by at least 25% from baseline; improvement of verbal fluency, visuospatial memory, and delayed recall
	Jorge et al., 2008 [103]	Type of study: prospective, randomized, sham-controlled Subjects: 92, randomized in active (48) and sham (44) groups; experiment 1: two groups of 15 patients each; experiment 2: 33 "real" patients and 29 sham patients Type of coil: figure-of-eight coil (7 cm diameter) Stimulation site: left DLPFC Stimulation frequency: 10 Hz Intensity: 110% rMT Length: 30 minutes Duration: 10 sessions (experiment 1), 15 sessions (experiment 2), 6 s period of stimulation, with a total of 20 trains separated by 1 min pauses, over 10 days Total number of pulses delivered: 12,000 (experiment 1); 18,000 (experiment 2)	Experiment 1: significant decrease in HDRS scores for real stimulation compared to sham; experiment 2: significant decrease in HDRS scores, increase in response rates, and remission rates for real stimulation compared to sham. Response rates to rTMS negatively correlated with age and positively correlated with higher frontal gray matter volume
	Robinson et al., 2009 [104]	Same patients and protocol of the experiment 2 of the study by Jorge and colleagues (2008) [103]. After rTMS or sham treatment, all responders were given citalopram for 9 weeks	Among the 33 "real" patients, 13 responded (>50% decrease in HDRS score); among them, 9 patients continued to be responders whereas the reaming 4 had a relapse of depression during the course of citalopram treatment
	Narushima et al., 2010 [105]	Type of study: prospective, randomized, sham-controlled	Significant difference in the response and remission rate of the HDRS scores

Table 2: Continued.

VCI subtype	Study, year	Study characteristics	Main findings
		Subjects: 43, randomized in "real" (32 patients) and "sham" (11 patients) groups Type of coil: figure-of-eight coil (7 cm diameter) Stimulation site: left DLPFC Stimulation frequency: 10 Hz Intensity: 110% rMT Duration: 10 sessions, 6 s period of stimulation, with a total of 20 trains separated by 1 min pauses, performed over 10 days Total number of pulses delivered: 12,000 (12 patients)–18,000 (31 patients)	between active and sham groups, in favor of the "real" stimulation group; increased low-theta power in the subgenual cingulate predicted the response to rTMS

Legend (in alphabetical order): DLPFC: dorsolateral prefrontal cortex; HDRS: Hamilton depression rating scale; M1: primary motor cortex; rMT: resting motor threshold; rTMS: repetitive transcranial magnetic stimulation; VCI: vascular cognitive impairment.

the relationship between excitability and plasticity in subcortical ischemic VaD, a cross-sectional study found that M1 had enhanced excitability in both AD and subcortical ischemic VaD patients, and more interestingly, M1 was plastically rearranged in both groups [92]. The results demonstrated indeed a functional cortical reorganization of all patients, with a slightly smaller frontal shift in the center of gravity for subcortical ischemic VaD compared to AD. A direct correlation between parameters of cortical excitability and those associated with the topographic shift of cortical maps was also noted [92]. Authors hypothesized that partially overlapping electrophysiological mechanisms probably act in the same manner in both VaD and AD, although they may differ both in location (subcortical vs. cortical) and origin (vascular vs. degenerative). Therefore, these disorders might share a common neurophysiological platform represented by a progressive neuronal loss in the motor areas in AD and a vascular disconnection in the white matter in subcortical ischemic VaD [115]. Eventually, these alterations will promote a functional brain rearrangement allowing to preserve motor programming and execution [84, 85].

Neurochemically, the reduction of rMT in both VaD and AD might represent a marker of impaired glutamatergic transmission, with an imbalance between non-NMDA and NMDA activity [116, 117]. Coherently, enhanced cortical excitability has been observed after the administration of an NMDA antagonist [118]. However, the facilitation of cortico-spinal outputs might also be caused by reduced intracortical inhibition [33]. Indeed, an increased GABA release may be a response to an overactivation of glutamate as part of the neuronal defense mechanisms leading to the compensation for excitotoxicity [119]. However, the studies here reviewed did not find significant changes of the TMS-related measures of inhibition, such as cSP, iSP, and SICI, while a significant SAI reduction was found in subcortical ischemic VaD [89]. In a different study, however, the reduction of SAI was noted in AD but not in VaD, apart from 25% of VaD patients that probably had a mixed dementia [88]. Even microbleeds in subcortical ischemic VaD might to have an impact on SAI-related cholinergic pathways, which was independent of the WMLs extent and ischemic stroke [91].

3.3. Vascular Depression. TMS studies are in line with the other findings in classifying vascular depression as a distinct nosologic entity, different from early-onset MDD [94]. In vascular depression, depressive symptoms, rather than signs of a primary disease status, are part of the wide spectrum of clinical presentations of the subcortical cerebrovascular disease [93]. Another difference between geriatric vascular depression and early-onset MDD is the enhancement of ICF observed only in former [93, 94]. According to the vascular depression hypothesis, this finding may imply that the disruption of the frontal-striatal circuits caused by vascular lesions may predispose, precipitate, or perpetuate a late-life depression [120].

However, from a neurophysiological perspective, very little is known on plasticity preserving cognitive functions in geriatric depression. By investigating the evolution of neurophysiological parameters in nondepressed patients with mild VCI and those with vascular depression, it has been shown that only nondepressed patients had a high level of ICF at the initial TMS evaluation [95]. At follow-up, a glutamate-related enhanced plasticity may have taken place in nondepressed patients that might be protective against cognitive deterioration, giving also cues on the possible role played by the late-life depression in the progression of VCI. Further, reduced rMT in both patient groups at follow-up points to the glutamatergic neurotransmission involvement. However, no specific change of neurophysiological parameter correlated with cognitive decline in depressed patients, suggesting that cognitive deterioration in vascular depression might be related to the subcortical lesion load or to the lack of compensatory cortical inputs [95].

3.4. CADASIL. The mutations in the *Notch3* gene on chromosome 19 causes CADASIL, that manifests with progressive cognitive decline till dementia, migraine, psychiatric disorders, and cerebral ischemic events. For this reason, it represents a genetic model of VaD that is interesting to study from a neuropsychologic and electrophysiological point of view [121].

In the first TMS study, a reduction in rMT and SAI [96] was found and attributed to the disruption of different

cortical-subcortical circuits caused by vascular lesions and locations [122], such those affecting the external capsule [123]. Regarding SAI, the significant reduction observed in both AD and CADASIL may be due to the involvement of different pathways, in that the L-3,4-dihydroxyphenylalanine (L-DOPA) administration was able to restore SAI only in AD [99], thus also providing therapeutic insights.

CADASIL patients also present an impaired sensory-motor plasticity induced by PAS [97]. Further, an association between WMLs load and lowered fractional anisotropy, along with an abnormal enhancement of LTP-like plasticity induced by PAS, has been observed particularly in the frontal commissural fibers. The authors' suggestion was that the increase in cortical plasticity might compensate the deterioration of cognitive and motor functions [97]. However, older patients with impaired cognition manifested opposite results, with a lower PAS-induced cortical plasticity, as well as a reduction of SAI and ICF [98]. In this study, a lower LTP-like plasticity in a stage of overt cognitive disorder may have failed in the compensatory mechanisms [98].

3.5. Repetitive TMS in Vascular Cognitive Impairment. Several rTMS studies, although methodologically heterogeneous, have shown that specific paradigms of stimulation might improve cognitive performance and have been proposed as a possible alternative to conventional neuroleptic therapy to behavioral symptoms of dementia. This is of particular interest because current pharmacological treatments suffer of significant limitations, such as nonspecific effects, insufficient tailoring to the individual, and moderate-to-severe adverse effects [124]. In this context, the target for an ideal rTMS treatment would be: (i) modulation of activity specifically in the targeted cortex, (ii) modulation of activity in a dysfunctional network, (iii) restoration of adaptive balance in a disrupted network, (iv) guiding plasticity for best outcome, and (v) suppression of maladaptive changes for functional advantage.

In a randomized controlled pilot study on patients with subcortical ischemic vascular disease and a clinical diagnosis of mild VCI, high-frequency rTMS over the left dorsolateral prefrontal cortex (DLPFC) induced a long-lasting improvement of the executive performance, likely due to an indirect activation of the midbrain monoaminergic neurons (dopamine) and/or of the brainstem (noradrenaline and serotonine) and their cortical and subcortical targets [100]. In the same patients, rTMS was able to alleviate depressive symptoms, suggesting a potential application even in individuals with vascular depression, although WMLs and global vascular risk factors were predictors of poor response [125].

Few years later, a randomized, controlled, crossover study on 7 mild VCI patients [101] stimulated the left DLPFC and the left M1 both at low- and high-frequency rTMS for 4 sessions (two at 1 Hz and two at 10 Hz). The authors found a significant improvement in the Stroop color-word interference test after the stimulation of the DLPFC but not the M1. An improvement was also noted in the digit symbol subtest of the revised Wechsler Adult Intelligence Scale after rTMS, regardless of the stimulation site [101].

3.6. Repetitive TMS in Vascular Depression. Based on the FDA approval for the treatment of drug-resistant MDD in adults [126] and according to the view that depressed patients exhibit a significant interhemispheric asymmetry in motor cortex excitability (i.e., lower excitability of the left hemisphere) [34], two main rTMS protocols, i.e., high-frequency rTMS (5-20 Hz) over the left DLPFC and low-frequency rTMS (1 Hz) on the right DLPFC [127], have been evaluated. The protocol using the high-frequency rTMS [128] reached a remission rate up of 15% in the "real" (treated) stimulation group with respect to 5% of the "sham" (simulated) stimulation group [129].

Globally, rTMS seems to be less effective in late-onset patients with geriatric depression [130, 131], probably due to the brain atrophy (especially in the frontal lobes) and ischemic WMLs (especially in the prefrontal areas) characterizing this age group, both disrupting the connections between DLPFC and subcortical areas underlying mood and affect control [132]. Nevertheless, an earlier analysis [133] did not find age as a significant predictor of response, whereas positive predictors were a shorter duration of the current depressive episode (<2 years) and the degree of treatment resistance (≤1 treatment failure vs. >1).

In the attempt of exploiting rTMS as a therapeutic option for vascular depression, a small open trial showed that 10 sessions of high-frequency rTMS applied over the left DLPFC improved not only verbal fluency, visuospatial memory, and delayed recall but also depressive symptoms [102]. In a larger prospective randomized sham-controlled study, high-frequency rTMS over the same cortical region successfully treated depressive symptoms and increased both response and remission rates [103]. These results suggested that rTMS may modulate both cognitive ability and depressive symptoms, probably by activating different but closely spaced neural networks. Preliminary findings were confirmed by two subsequent randomized trials, one that combined rTMS with citalopram treatment [104] and one by using electroencephalography (EEG) in the follow-up period [105]. The studies showed significant differences in response and remission rates of depressive symptoms between active and sham groups, favoring the "real" stimulation. The second study also found that the increased "low-theta" band power in the subgenual cingulate cortex predicted the response to rTMS [105].

Finally, low-frequency rTMS over the right DLPFC was tested in a patient with drug-resistant depression and cerebral amyloid angiopathy, which is a chronic neurovascular disorder characterized by a progressive amyloid-β fibril deposition within the wall of cerebral blood vessels, eventually leading to hemorrhagic events and dementia. Stimulation intensity was set to 110% of the rMT, and rTMS was applied at 1 Hz for 1,600 pulses per day for 3 weeks. A long-lasting decrease in depression rating scales was noted, thus opening the way for the treatment of depression in other forms of cerebrovascular and degenerative diseases [134].

3.7. Translational Considerations. To date, the prediction of dementia onset and progression is beyond the possibilities of conventional tools. However, differently from AD and

other degenerative disorders, VaD can be slowed, delayed, or even avoided through a careful prevention and control of vascular risk factors [135]. Besides the prevention of vascular accidents, maintaining the functional status in the elderly is a further key factor in the prevention and management of VCI.

Because of the VCI's heterogeneous construct, the selection of appropriate outcome measures to employ in pharmacological trials is of particular importance. In this context, the early discovery of new therapeutic targets would lead to a better prevention and treatment of VaD, and accordingly with the reviewed literature, TMS can be of help [33]. An enhancement of cortical plasticity might be induced to counteract cognitive decline, and the evaluation of where and how much this happens in different patients' subpopulations may shed light on the pathophysiological bases of decline or preservation of cognition [115].

Although a single TMS measure cannot be used to diagnose VCI, collectively the parameters of interest may act as footprints of VCI pathophysiology. Moreover, TMS can help to identify different profiles of cortical excitability for VCI subtypes and for the prediction of the "brain at risk" to convert into an overt VaD [28, 31, 95]. These findings will also support the study design of trials to test new drugs and novel nonpharmacological approaches. Finally, clinicians can exploit TMS in patients with overt dementia for the selection of the response to specific drugs [110], and the efficacy of treatment can be maximized by selecting the patients on the basis of putative neurophysiological markers.

Neurotrophins have an important role in the response to vascular damage and in stroke recovery [136, 137], and their release and modulation may also be behind the mechanisms of action of noninvasive brain stimulation in dementia. Several murine models of VaD have been used for testing rTMS [138], showing that low-frequency rTMS positively impact on cognitive deficit by upregulating the release of the hippocampal brain-derived neurotrophic factor (BDNF) and the expression of the NMDA glutamate receptor [139]. A different study found that increased expression in the *Bcl-2* gene and a decrease in the *Bax* gene led low-frequency rTMS to be effective in learning and memory, as well as in the protection of pyramidal cells from apoptosis and in the promotion of hippocampal synaptic plasticity [140]. Moreover, rTMS significantly improved learning and memory and increased acetylcholinesterase and choline acetyltransferase activity, the density of cholinergic neurons, and the number of BDNF-immunoreactive cells at the level of hippocampal CA1 region [141]. Finally, in VaD rats, synaptic plasticity showed to be synergic with mesenchymal stem cells transplantation and with the promotion of autophagy [142]. However, the effectiveness of rTMS as VCI disease-modifying therapy in humans deserves further translational considerations, larger samples size, and well-controlled investigations [143].

Similarly, the clinical efficacy of rTMS on the cognitive aspects of vascular depression is still a matter of debate. It cannot be excluded, indeed, that cognitive improvement might be the consequence of an indirect effect on depressive symptoms rather than an improvement of cognition *per se*. In this context, while findings on rTMS in vascular depression are still limited and a conclusive evidence is yet to be reached, rTMS data in MDD (which is often associated with cognitive changes, especially executive dysfunction) are much more robust [144]. In MDD, the treatment-induced response did not seem to be directly related to a relief from depression or other treatment variables, thus suggesting that improvement of cognition and mood may follow different mechanisms [145]. Based on earlier controlled studies [146–148], improvement in both verbal fluency and visuospatial memory suggests that rTMS may enhance specific aspects of cognition independently from positive mood changes through a general alerting effect or a learning facilitation [102]. Moreover, since previous investigations did not find significant correlations between cognitive functioning and depression scores [149–151], it has been proposed that rTMS might independently modulate cognitive abilities and depression symptoms by activating different neural pathways and brain regions. In addition, in a pilot study on treatment-resistant depressed patients [151], left frontal high-frequency rTMS was associated with better performance of tests evaluating frontal lobe abilities and reduction in depression severity. The authors hypothesized that the cognitive improvement could be due to a direct or indirect (i.e., transsynaptic) modulation of the DLPFC [151], probably secondary to the activation of monoaminergic neurons in the midbrain (dopamine) or in the brainstem (noradrenaline and serotonin) and their cortical and subcortical targets [152, 153].

Lastly, it was demonstrated that rTMS not only improved executive dysfunction in MDD patients but also restored the interhemispheric asymmetry of rMT and ICF, thus implying that specific electrocortical changes may correlate to executive functions, both before and after treatment [154]. Although the pattern of motor cortex excitability in vascular depression differs from that previously reported in MDD and is similar to that of patients with subcortical vascular disease [28], the clinical presentations of these patients are similar, i.e., psychomotor retardation, difficulties at work, apathy, lack of insight, and executive dysfunction. This may suggest that, in vascular depressed patients, the enhancement of ICF could play a compensatory glutamate-mediated role in response to vascular damage of the frontal cortical-subcortical circuits implicated in mood-affect regulation and cognition [94]. Nevertheless, as mentioned above, the effects of rTMS on cognitive functioning can depend on additional factors (e.g., the paradigms used and the parameters studied), and it is not always observed [155], thus warranting further evidence in both MDD and vascular depression.

At this stage, it is not certain that the findings of the studies we reviewed have a direct impact in the daily decision-making algorithm of VCI patients' care, although they suggest that TMS can help to screen populations at risk. Once the population at risk is identified, a careful prevention and vascular risk factors control can be achieved more easily. Further longitudinal studies combining TMS with other neurophysiological techniques, such as high-density EEG and multimodal evoked potentials, as well as with advanced structural and functional neuroimaging (such as diffusion tensor imaging and functional MRI) and serum or CSF analysis will clarify the impact of cognitive and mood deficits on VCI plasticity.

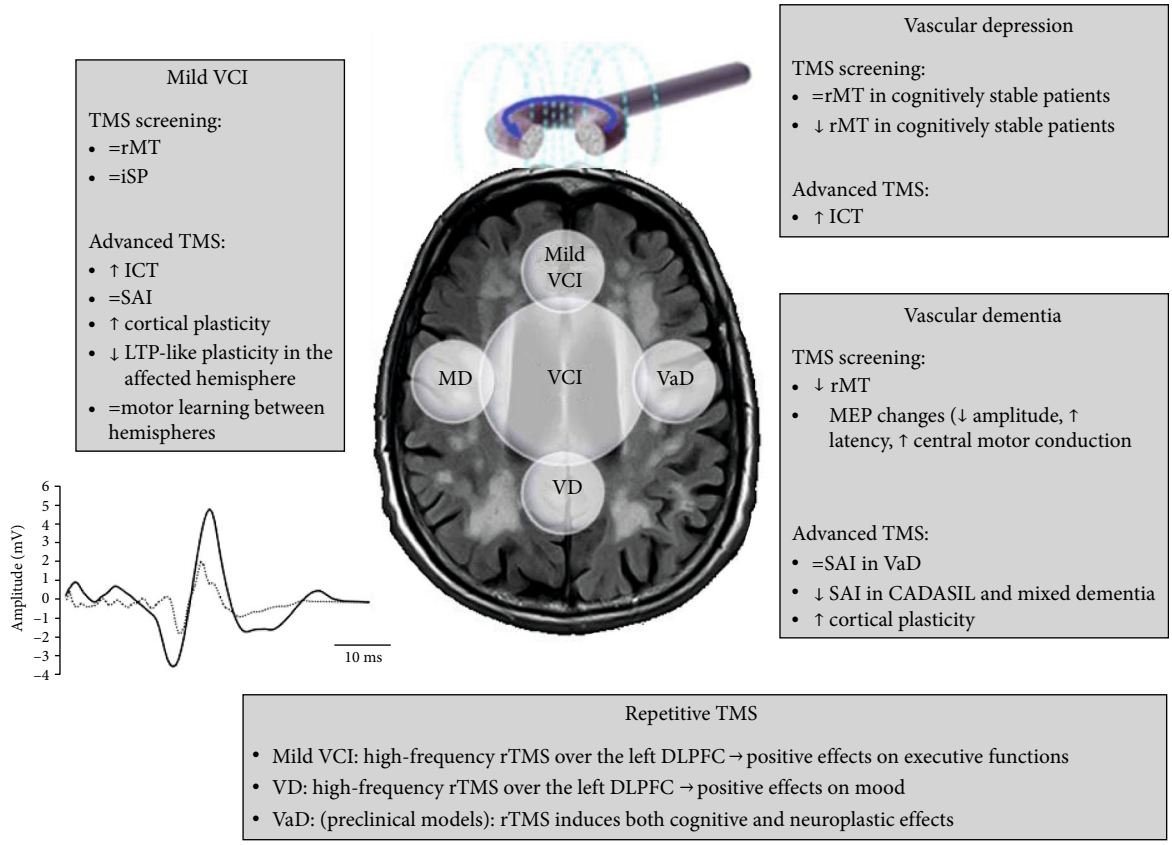

Figure 3: TMS findings, proposed diagnostic algorithm, and main rTMS effects in VCI. Legend (in alphabetic order): CADASIL: cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; DLPFC: dorsolateral prefrontal cortex; ICF: intracortical facilitation; iSP: ipsilateral silent period; LTP: long-term potentiation; MD: mixed dementia; MEP: motor evoked potential; rMT: resting motor threshold; rTMS: repetitive transcranial magnetic stimulation; SAI: short-latency afferent inhibition; TMS: transcranial magnetic stimulation; VaD: vascular dementia; VCI: vascular cognitive impairment; VD: vascular depression.

Figure 3 illustrates the TMS findings in VCI, proposes a diagnostic algorithm, and summarizes the main rTMS effects.

3.8. Critical Aspects, Possible Solutions, and Future Research. A major limitation in the implementation of the studies employing noninvasive brain stimulation in VCI is the relatively small sample sizes that make the generalization of these results to large populations troublesome. The same holds for the difficulty to recruit enough elderly healthy controls without neuroimaging evidence of cerebrovascular disease.

Second, the relatively low spatial resolution of TMS often determines the lack of systematic correlation between the pattern of cortical excitability and the anatomical distribution and severity of vascular lesions. Combining TMS with advanced imaging, neuronavigational systems, and other electrophysiological techniques may overcome this issue.

Third, although the TMS-related measures of cortical excitability are sensitive to the "global weight" of many neurotransmitters, so far we do not have more detailed information linking TMS findings with specific cognitive or behavioral changes [54, 111]. In this context, hypothesizing the presence a specific "signature" characteristic of VCI patients could be risky given the paucity of previous data and the difficulty that similar approaches are encountered in other dementing conditions, such as the non-AD dementias [33]. Additionally, even in the absence of evident motor deficit, vascular lesions significantly contribute to degenerative dementias and their progression. Therefore, it cannot be excluded that some of the enrolled patients had a mixed dementia rather than a pure VaD. In other words, TMS profile alone is not currently capable of distinguishing VaD from AD [115].

It should also be noted that antithrombotic agents, oral antidiabetic therapy, antihypertensive drugs, and statins, commonly prescribed to elders, might affect the measures of cortical excitability and their response to rTMS treatments [156, 157]. Thus, both TMS and rTMS studies need to consider this possible confounding factor.

Finally, for an adequate definition of sensitivity and specificity, the individual TMS measures in all patients and controls would be necessary. Besides, the estimation of the number of false positives would require an independent follow-up allowing the assessment of the cognitive status. Those requirements have been met only by a few studies, so that the next applications of TMS in VCI need methodological improvements and higher standardization levels.

Regarding rTMS, it is relatively expensive and requires technical expertise. Moreover, the magnetic coil must to be held still, and sham stimulation and operator blindness are often difficult. The majority of reported investigations are open-label or uncontrolled, and the treatment response could be affected by changes in brain morphology (e.g., cortical atrophy or CSF distribution). Moreover, determining the most appropriate target for stimulation is often challenging, and inferring to what extent cortical response characteristics of the motor system are representative of other brain areas is often speculative. Finally, there is a wide range of TMS parameters and rTMS settings that need to be considered in these applications.

Possible solutions may consist of [158]: (i) fully report of the results all rTMS trials, including negative findings; (ii) more studies in healthy individuals or in those with mild disease, thus allowing finessing of stimulation parameters and establishing the tolerability of protocols; (iii) further studies on the etiological models of dementia, including preclinical ones, thus aiding the choice of stimulation site and other technical set up; (iv) optimization of the treatment efficacy through methods of stratification, where patients are selected on the basis, for instance, of neuropsychological, electrophysiological, or genetic markers; and (v) use of novel methodological factors that can increase the stimulation efficacy, as well as the combination of rTMS with objective outcome measures (e.g., those derived from EEG, CSF, or MRI).

4. Conclusions

Overall, there is a mounting interest towards new diagnostic and therapeutic tools for cognitive assessment and rehabilitation in dementia, including VCI. Current data, although obtained from heterogeneous studies, have revealed that TMS and rTMS can provide, respectively, valuable diagnostic clues and induce beneficial effects on some cognitive domains and neuropsychiatric manifestations. Challenges still exist in terms of appropriate patient selection and optimization of the stimulation protocols. Recent findings from animal models are exciting, but their clinical significance needs to be validated. Together with the clinical exam, psychocognitive assessment, and neuroimaging, a systematic TMS evaluation of VCI patients can aid the diagnostic process, enhance the therapeutic arsenal, and predict the prognosis.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Mariagiovanna Cantone and Giuseppe Lanza contributed equally to this work.

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