Metaplasticity: A Promising Tool to Disentangle Chronic Disorders of Consciousness Differential Diagnosis

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The extent of cortical reorganization after brain injury in patients with Vegetative State /Unresponsive Wakefulness Syndrome (UWS) and Minimally Conscious State (MCS) depends on the residual capability of modulating synaptic plasticity. Neuroplasticity is largely abnormal in patients with UWS, although the fragments of cortical activity may exist, while patients MCS show a better cortical organization. The aim of this study was to evaluate cortical excitability in patients with disorders of consciousness (DoC) using a transcranial direct current stimulation (TDCS) metaplasticity protocol. To this end, we tested motor-evoked potential (MEP) amplitude, short intracortical inhibition (SICI), and intracortical facilitation (ICF). These measures were correlated with the level of consciousness (by the Coma Recovery Scale-Revised, CRS-R). MEP amplitude, SICI, and ICF strength were significantly modulated following different metaplasticity TDCS protocols only in the patients with MCS. SICI modulations showed a significant correlation with the CRS-R score. Our findings demonstrate, for the first time, a partial preservation of metaplasticity properties in some patients with DoC, which correlates with the level of awareness. Thus, metaplasticity assessment may help the clinician in differentiating the patients with DoC, besides the clinical evaluation. Moreover, the responsiveness to metaplasticity protocols may identify the subjects who could benefit from neuromodulation protocols.

Keywords: Metaplasticity; TDCS; UWS; MCS; consciousness.

1. Introduction

The human brain networks have a remarkable property to maintain the stability of neural functions and their synaptic excitability and plasticity through homeostatic mechanisms.¹ This finely-tuned plasticity regulation keeps neural activity within a dynamic range concerning many functions, including brain damage recovery.^{2–5} This avoids the development of uncontrolled and maladaptive plasticity (including the overexpression of long-term potentiation — LTP — and depression — LTD — mechanisms).

These homeostatic mechanisms include metaplasticity, a term originally proposed by the theoretical physicist Cooper in 1982.⁶ Metaplasticity adjusts the ongoing threshold for LTP and LTD induction depending on the previous synaptic activity,^{1,2,7–12} and balancing different types of influencing factors, including acute stress and related hormones, and addicting factors,^{13–16} thus altering the magnitude and duration of the subsequent synaptic events. In this way, metaplasticity avoids synaptic activity saturation and endows neural networks

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with an ongoing ability to respond to an ever-changing environment and to update continuously the information storage capacity. These properties are fundamental concerning several functions, including consciousness^{17,18} and brain damage recovery.^{2-7,19}

Metaplasticity can be experimentally investigated following two consecutive neuromodulation protocols: priming and conditioning.³ Generally speaking, the former (including noninvasive brain stimulation) modifies the after-effects of the latter but the direction of such effects depends on the targeted synapses. When priming and conditioning focus on the same synapses, metaplasticity occurs homosynaptically by the means of mechanisms dependent on the *N*-methyl-*D*-aspartate receptor (LTP prevention) or metabotropic glutamate receptor (LTP fostering).²⁰ Otherwise, metaplasticity occurs heterosynaptically, leading to the LTP inhibition and LTD facilitation.^{21,22}

Among the noninvasive brain stimulation tools, Transcranial Direct Current Stimulation (TDCS) has been proposed to interfere with LTP and LTD-like plasticity mechanisms.^{23,24} In particular, TDCS, which consists of low constant currents delivered through small surface electrodes, can either depolarize (anodal TDCS) or hyperpolarize (cathodal TDCS) neuron's resting membrane, thereby increasing or decreasing the cortical excitability, respectively.²⁵ Therefore, TDCS is most suitable to study metaplasticity.

The patients suffering from chronic Disorders of Consciousness (DoC), including Minimally Conscious State (MCS) and Vegetative State/Unresponsive Wakefulness Syndrome (UWS), show a large-scale cortico-thalamo-cortical connectivity breakdown and cortical disexcitability, in the reason of the loss of inhibitory tone from the thalamus due to the thalamo-cortical damage.^{26,27} The degree of cortico-thalamo-cortical connectivity deterioration correlates with the consciousness level and the outcome of DoC individuals.²⁸ Nonetheless, some patients with DoC show cortical excitability and connectivity during noninvasive brain stimulation higher than those expected based on the clinical data.²⁹⁻³¹ In fact, some patients may be misdiagnosed as suffering from UWS.³² In other words, some patients with DoC do manifest a behavior similar to UWS, but electrophysiologically they are MCS. Thus, these

patients are unable to communicate behaviorally, probably due to the specific sensory-motor integration deficits.^{29,30} Therefore, we may hypothesize that noninvasive brain stimulation may unmask residual networks subtending awareness in some patients clinically defined as UWS. However, patients with DoC show a maximal facilitation of excitability and plasticity mechanisms yet at the baseline, in an attempt to regain network functions.^{33,34} However, such mechanisms work in a maladaptive manner, i.e. persistently and unadaptably.³⁵ Therefore, some patients with DoC may not show a response to simple noninvasive brain stimulation paradigms, as they cannot cross the threshold for plasticity modifications.²⁸ Consequently, such patients remain misdiagnosed. Thus, the use of more complex noninvasive brain stimulation protocols, including those concerning metaplasticity, could be of some help, since complex protocols could entrain more complex networks and plasticity mechanisms. This could be important in keeping with the sensibility of metaplasticity to stressing factors, which are very common in patients with DoC.^{13-18,44} In this way, it could be possible to unmask the residual connectivity and plasticity properties that may support covert awareness. We studied the effects of a priming and a conditioning TDCS on the premotor and motor cortex metaplasticity of the patients with DoC and correlated clinical and electrophysiological measures to better differentiate the patients with DoC.

2. Methods

2.1. Subjects

We enrolled 40 patients with DoC (19 with MCS and 21 with UWS) following hypoxic-ischemic or traumatic brain damage, who were compared with 25 healthy control (HC) individuals (14 females and 11 males; mean age 52 ± 5 years). The patients met the criteria for vegetative state and MCS diagnosis.^{36,37} The detailed demographic and clinical characteristics are reported in Table 1. The exclusion criteria were pre-existing severe neurological or systemic diseases; any critical condition; administration of other modifying cortical-excitability drugs than *L*-Dopa, analgesics, baclofen, and anti-epileptic drugs; epileptic history; the presence of electric/electromechanical devices within head and neck; the presence of electroencephalographic suppression-burst pattern; the

Table 1. Clinical-demographic characteristics.

DoC	Etiology, gender, age, onset	MRI	CRS-R							
			Total	A	V	M	OM	C	Ar	Treatment
MCS (<i>n</i> = 19)	<i>T, F</i> , 70 y, 13 m	Fb_h	20 ± 4	4 ± 1	4 ± 1	5 ± 1	3 ± 1	1	3 ± 1	1
	<i>A, M</i> , 57 y, 9 m	WMH	19 ± 4	4 ± 1	4 ± 1	5 ± 1	2 ± 1	1	3 ± 1	1 + 2
	<i>T, F</i> , 72 y, 6 m	FP_h	18 ± 4	4 ± 1	3 ± 1	4 ± 1	3 ± 2	1	3 ± 1	1 + 3
	<i>T, M</i> , 47 y, 12 m	FP_h	18 ± 4	3 ± 1	4 ± 1	5 ± 1	2 ± 1	1	3 ± 1	
	<i>T, M</i> , 33 y, 18 m	multiple_h	12 ± 2	2 ± 1	2 ± 1	3 ± 1	2 ± 1	1	2 ± 1	2
	<i>A, M</i> , 51 y, 18 m	WMH	10 ± 2	2 ± 1	2 ± 1	3 ± 1	1 ± 1	1	1 ± 1	1 + 2
	<i>T, F</i> , 44 y, 3 m	F_h	9 ± 4	2 ± 1	2 ± 1	2 ± 1	1 ± 1	1	1 ± 1	1 + 3
	<i>T, F</i> , 43 y, 8 m	Fb_h	20 ± 4	4 ± 1	4 ± 1	5 ± 1	3 ± 2	1	3 ± 1	1
	<i>A, M</i> , 48 y, 9 m	WMH	19 ± 4	4 ± 1	4 ± 1	5 ± 1	2 ± 2	1	3 ± 1	1 + 2
	<i>T, F</i> , 50 y, 7 m	FP_h	18 ± 4	4 ± 1	3 ± 2	4 ± 2	3 ± 3	1	3 ± 1	1 + 3
	<i>A, F</i> , 59 y, 21 m	WMH	18 ± 1.7	4 ± 1	4 ± 1	5 ± 1	1 ± 1	1	3 ± 1	1
	<i>V, F</i> , 65 y, 29 m	F_h	13 ± 1.0	3 ± 1	2 ± 1	3 ± 1	1 ± 3	1	3 ± 1	1 + 2
	<i>T, M</i> , 45 y, 26 m	Fb_h	10 ± 0.8	3 ± 1	2 ± 2	2 ± 2	1 ± 1	1	1 ± 1	1 + 3
	<i>V, M</i> , 56 y, 32 m	P_IS	14 ± 1.5	3 ± 1	2 ± 2	5 ± 2	2 ± 1	1	1 ± 1	
	<i>T, M</i> , 69 y, 17 m	FP_h	12 ± 0.9	2 ± 1	3 ± 1	3 ± 1	1 ± 3	1	2 ± 1	2 + 3
	<i>V, F</i> , 41 y, 38 m	SAH	15 ± 1.6	2 ± 1	4 ± 2	5 ± 1	2 ± 3	1	1 ± 1	1 + 2 + 3
	<i>A, M</i> , 53 y, 35 m	WMH	14 ± 1.2	3 ± 1	4 ± 2	2 ± 2	3 ± 1	1	1 ± 1	1 + 3
	<i>V, M</i> , 62 y, 28 m	BG_h	18 ± 1.1	3 ± 1	4 ± 2	4 ± 2	3 ± 1	1	3 ± 1	
	<i>T, F</i> , 75 y, 13 m	PO_h	15 ± 1.4	3 ± 1	4 ± 1	4 ± 2	1 ± 3	1	2 ± 1	1 + 2 + 3
	mean ± SD	10T, 5A, 4V, 8F, 11M, 55 ± 12 y, 18 ± 11 m	15 ± 1	3 ± 1	3 ± 1	4 ± 1	2 ± 2	1	2 ± 1	
UWS (<i>n</i> = 21)	<i>A, F</i> , 62 y, 19 m	WMH	6 ± 2	2 ± 1	1 ± 0.5	1 ± 0.5	0.1 ± 0.1	0	2 ± 0.5	
	<i>A, F</i> , 43 y, 6 m	WMH	6 ± 2	2 ± 1	1 ± 1	2 ± 1	0.1 ± 0.1	0	1 ± 1	1
	<i>T, M</i> , 48 y, 11 m	multiple_h	7 ± 3	1 ± 1	1 ± 0.5	2 ± 1	1 ± 1	0	2 ± 1	3
	<i>T, F</i> , 54 y, 15 m	DAI + F_h	5 ± 2	1 ± 1	1 ± 1	1 ± 0.5	0.5 ± 0.2	0	1 ± 1	2 + 3
	<i>T, M</i> , 38 y, 12 m	DAI + FP_h	5 ± 2	1 ± 1	1 ± 1	1 ± 1	0.5 ± 0.2	0	1 ± 1	3
	<i>A, F</i> , 38 y, 6 m	WMH	7 ± 3	1 ± 0.2	2 ± 0.1	1 ± 0.5	0.5 ± 0.2	0	2 ± 0.5	3
	<i>T, M</i> , 45 y, 6 m	DAI	6 ± 2	1 ± 1	1 ± 1	1 ± 1	0.5 ± 0.5	0	2 ± 0.5	1 + 3
	<i>A, F</i> , 47 y, 11 m	WMH	6 ± 2	2 ± 1	1 ± 0.5	1 ± 0.5	0.1 ± 0.2	0	2 ± 0.6	
	<i>A, F</i> , 42 y, 13 m	WMH	6 ± 2	2 ± 1	1 ± 0.2	2 ± 1	0.1 ± 0.2	0	1 ± 2	1 + 3
	<i>T, M</i> , 43 y, 9 m	multiple_h	6 ± 2	1 ± 1	1 ± 0.6	1 ± 1	1 ± 2	0	2 ± 2	
	<i>T, F</i> , 46 y, 12 m	DAI + F_h	5 ± 2	1 ± 1	1 ± 1	1 ± 0.6	0.5 ± 0.5	0	1 ± 1	2
	<i>V, F</i> , 68 y, 19 m	FP_IS	6 ± 0.6	1 ± 1	1 ± 1	2 ± 1	1 ± 2	0	1 ± 2	
	<i>A, M</i> , 42 y, 34 m	WMH	5 ± 0.4	1 ± 1	1 ± 1	1 ± 1	1 ± 2	0	1 ± 1	1 + 3
	<i>A, M</i> , 63 y, 19	WMH	5 ± 0.8	2 ± 1	1 ± 1	1 ± 1	0 ± 1	0	1 ± 1	1 + 3
	<i>V, F</i> , 62 y, 21 m	FTP_IS	6 ± 0.6	2 ± 1	1 ± 1	1 ± 1	1 ± 2	0	1 ± 2	2
	<i>A, F</i> , 52 y, 19 m	WMH	7 ± 0.8	1 ± 1	1 ± 1	2 ± 1	1 ± 1	0	2 ± 2	1
	<i>A, M</i> , 58 y, 28 m	WMH	6 ± 0.6	1 ± 1	1 ± 1	1 ± 1	1 ± 2	0	2 ± 1	
	<i>V, F</i> , 70 y, 18 m	BG_h	5 ± 0.4	1 ± 1	1 ± 1	1 ± 1	1 ± 2	0	1 ± 1	1
	<i>T, F</i> , 60 y, 14 m	SAH	5 ± 0.4	1 ± 1	1 ± 1	1 ± 1	1 ± 2	0	1 ± 2	2 + 3
	<i>T, M</i> , 49 y, 20 m	multiple_h	4 ± 0.5	1 ± 1	1 ± 1	1 ± 1	0 ± 1	0	1 ± 2	1
	<i>V, F</i> , 70 y, 39 m	TP_IS	5 ± 0.8	2 ± 1	1 ± 1	1 ± 1	0 ± 1	0	1 ± 1	1 + 2 + 3
	mean ± SD	8T, 9A, 4V, 12F, 9M, 52 ± 11 y, 17 ± 8 m	6 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1	0	1 ± 1	
Group comparison			< 0.001	< 0.001	< 0.001	< 0.001	< 0.001		0.1	

Note: L-Dopa; 2 baclofen; 3 anti-epileptic drugs; A anoxic; b basal; BG basal ganglia; CRS-R baseline coma recovery scale-revised (A auditory, V visual, M motor, OM oromotor, C communication, Ar arousal); DAI diffuse axonal injury; DoC disorder of consciousness; F female; F frontal; h hemorrhagic lesion; IS ischemic stroke; M male; MRI magnetic resonance imaging; NS nonsignificant; O Occipital; P parietal; SAH subarachnoid hemorrhage; T temporal; T traumatic; V vascular; WMH white matter hyper-intensity. Patients who are only apparently UWS are marked in light gray.

lack of motor-evoked potential (MEP) from right first dorsal interosseous muscle. The present study was approved by the Local Ethics Committee and written informed consent was obtained either from HC or the legal guardian of each patient.

2.2. Experimental procedure

We determined the effects on resting motor threshold, MEP amplitude,^{40–45} short intracortical inhibition (SICI), and intracortical facilitation (ICF) strength (according to the Kujirai paired-pulse method⁴⁵), when applying the priming TDCS before the conditioning TDCS both to the left primary motor cortex, and when applying the priming TDCS to the left premotor cortex before the conditioning TDCS to primary motor cortex. These two brain areas were chosen to investigate primary motor cortex metaplasticity and the interregional metaplasticity between premotor and primary motor cortex (which reflects a functional connection).⁴⁶ Both anodal and cathodal TDCS were tested in all possible priming and conditioning combinations within primary motor cortex and between premotor and primary motor cortex. In addition, a priming

sham-TDCS preceded a conditioning cathodal TDCS in primary motor and premotor cortex. The time interval between the priming and conditioning TDCS was set as 3 min.^{38,39} We did not test other priming–conditioning intervals to avoid a highly fatiguing paradigm in such frail patients. Each subject followed all the protocols in different sessions, at every two days, while the order of sessions was kept random. Each parameter (MEP amplitude, SICI, and ICF strength) was tested before (PRE), and after 10 (*T*₁), 30 (*T*₂), and 60 min (*T*₃) of the TDCS application. The experimental procedure with stimulation setup details is summarized in Fig. 1.

2.3. Statistical analysis

Student's *t*-tests were performed to determine whether the baseline parameters differed significantly. In all conditions, the normal distribution of the data was evaluated with the Kolmogorov–Smirnov test (all *p* > 0.2).

The effects of TDCS (delivered on primary motor cortex and on premotor and primary motor cortex) on each electrophysiological parameter (MEP, SICI, and ICF) were measured by three-way

Fig. 1. Experimental design. TDCS was delivered through a BrainStim battery-driven electric stimulator (E.M.S., Bologna, Italy) wired to a pair of surface rubber electrodes (35 cm²) inside a saline-soaked sponge, and arranged according to the type of conditioning (*c* cathode, black electrode; *a* anode, red electrode; PMc premotor cortex; M1 primary motor cortex). Currents were applied at 1mA (current density ~ 0.03 mA/cm²) for 10 min, with a 30 s ramping up/down period (this setup has been shown to be painless and strong enough to induce stable effects on motor cortex excitability). The other electrode was placed above the right orbit since this arrangement is known to result in significant excitability changes of the cortex. In the sham TDCS, the current was switched off after 30 s. Motor cortex excitability was tested through single-pulse TMS delivered on the motor hot-spot of the right FDI by using a figure-of eight magnetic coil (diameter of one winding 9 cm) wired to a Magstim 200 magnetic stimulator (Whiteland, Dyfed, UK). The coil was held tangentially to the skull, with the handle pointing backward and laterally at 45° from the midline. The optimal coil position was defined as the site where TMS stimulation resulted consistently in the largest MEP. We recorded 10 MEPs induced by single TMS pulses set at a stimulator output intensity of 120% of resting motor threshold, which were intermingled with 15 SICI and 15 ICF interactions in a single trial, at a frequency of 0.2 Hz. SICI and ICF were determined similarly to the Kujirai paired-pulse method. The intensity of the conditioning stimulus was set at a stimulator output intensity of 70% of resting motor threshold, and at an inter-stimulus interval of 2 ms and 12 ms, respectively. Such intensities and intervals were kept constant throughout the experiment. Mean amplitude of the conditioned MEP was expressed as the percentage of unconditioned MEP amplitude and was taken as a measure of corticospinal excitability.

repeated-measures ANOVA with *priming* (five levels: anodal–anodal, cathodal–cathodal, anodal–cathodal, cathodal–anodal, and sham–cathodal) and *time* (four levels: T_{PRE} , T_1 , T_2 , and T_3) as within-subject factors, and *group* (three levels: HC, MCS, and UWS) as between-subject factor. A p -values < 0.05 was considered significant. Depending on the significance of F -value, appropriate follow-up ANOVAs with the factors time and priming, and with the factors time, with *post-hoc t*-test (Bonferroni) were performed to explore the strength of main effects and the patterns of interaction between the experimental factors. In order to understand how large the differences between MCS and UWS were, we calculated the effect size Cohen's d . Given the relatively small sample size, we applied Hedge g correction to the biased effect size estimate. η^2 and λ were reported as well. Finally, the sensitivity and specificity of the electrophysiological measures employed to distinguish accurately between MCS and UWS were calculated by measuring the Area Under the Receiver Operating Characteristic Curve (AUC).

All data are given as mean or percent changes of the unconditioned MEP amplitude $\pm$ SD (i.e. the baseline MEP without TDCS or paired-pulse TMS paradigm). A Spearman's correlation analysis was carried out to assess the total correlation between clinical-demographic and electrophysiological parameters in the patients with DoC. Within- and between-group correlations were computed when the total correlation was nonsignificant.

3. Results

3.1. Baseline findings

No significant difference in the clinical-demographic parameters between the DoC groups, except for the CRS-R score, was found (Table 1). There was no significant difference between the resting motor threshold (52–60% in each group) and MEP amplitude (0.42–0.71 mV in each group). In the HC group, SICI was low (MEP amplitude decrease), whereas ICF caused an increase in MEP amplitude (Figs. 2 and 3). On the contrary, individuals with DoC showed a tonically high cortical excitability, which was more evident in the patients with UWS than those with MCS, as shown by high SICI and ICF values (Figs. 2 and 3).

3.2. Primary motor cortex metaplasticity

All the subjects completed the experimental procedure without any side effect, beside a very mild, seldom tingling sensation beneath the cathode/anode electrode in the HC individuals was reported. ANOVA showed that that TDCS significantly influenced cortical excitability (time $\times$ group $\times$ priming $\times$ parameter $F_{(48,2976)} = 4$, $p < 0.001$; $\lambda = 193$, $\eta^2 = 0.9$). In fact, we found a MEP amplitude and SICI strength modulation in HC and MCS individuals, while none of the UWS subjects (but three, n . 3, 16, and 19) showed excitability changes (Table 2). ICF was not influenced by any TDCS priming. In particular, we observed a significant increase in cortical excitability as shown by MEP amplitude increase when using cathodal–cathodal and cathodal–anodal TDCS in HC and MCS subjects and by SICI increase when using cathodal–anodal TDCS in HC (Fig. 2). Conversely, cortical excitability decreased, as indexed by MEP amplitude decrease, when using anodal–anodal and anodal–cathodal TDCS in HC, and by SICI decrease when using anodal–cathodal TDCS in HC and patients with MCS (Fig. 2). All these changes were detectable up to T_2 and were greater in magnitude in MCS than in HC individuals (Fig. 2), whereas the patients with UWS showed no changes. Nonetheless, only three patients with UWS (n . 3, 16, and 19) had a cortical facilitation/inhibition that was similar to that shown by the patients with MCS (Fig. 4). ICF did not show significant changes. Sham–cathodal TDCS in HC individuals induced a slight, nonsignificant SICI decrease without any effect on MEP and ICF magnitude, whereas it was ineffective in both DoC groups.

3.3. Premotor cortex–primary motor cortex metaplasticity

ANOVA showed that that TDCS significantly influenced cortical excitability (time $\times$ group $\times$ priming $\times$ parameter $F_{(48,2976)} = 2.6$, $p < 0.001$; $\lambda = 123$, $\eta^2 = 0.9$). In fact, we found a MEP amplitude and SICI and ICF strength modulation in HC and MCS individuals, while none of the UWS subjects (but three, n . 3, 16, and 19) showed excitability changes (Table 3). In particular, we observed a significant increase in premotor–motor excitability in HC and

Fig. 2. Primary motor cortex TDCS effects onto MEP amplitude and SICI and ICF strength in the three groups of participants. Error bars refer to SD. * indicates a significant change at T1 and T2 as compared to PRE (baseline).

Note: HC healthy subjects, UWS Unresponsive Wakefulness Syndrome, MCS Minimally Conscious State, aa both-anodal, ac anodal-cathodal, ca cathodal-anodal, cc both-cathodal.

MCS when using cathodal-anodal TDCS as indexed by MEP amplitude, SICI, and ICF increase (Fig. 3). Conversely, premotor-motor excitability decreased when anodal-anodal TDCS was used, as shown by MEP amplitude and SICI decrease in HC and MCS (Table 3). All these changes were detectable up to T2 and were greater in the patients with MCS than HC individuals, whereas patients with UWS showed no changes. Only three patients with UWS (n . 3, 16, and 19) had a premotor-motor facilitation/inhibition

that was similar to the one shown by the individuals with MCS (Fig. 4). Sham-cathodal TDCS showed the same outcome; a slight effect on MEP amplitude was found in primary motor cortex metaplasticity paradigm without any effect on SICI and ICF.

3.4. Clinical-electrophysiological correlations

We found a significant total correlation between the CRS-R and the overall TDCS-induced SICI changes,

Fig. 3. Premotor cortex–primary motor cortex TDCS effects onto MEP amplitude and SICI and ICF strength in the three groups of participants. Error bars refer to SD. * indicates a significant change at T_1 and T_2 as compared to PRE (baseline).

Note: HC healthy subjects, UWS Unresponsive Wakefulness Syndrome, MCS Minimally Conscious State, *aa* both-anodal, *ac* anodal–cathodal, *ca* cathodal–anodal, *cc* both-cathodal.

i.e. the higher was the CRS-R score, the greater was the SICI magnitude modulation (i.e. the reduction of SICI at T_1 , when the effect was stronger) ($r = -0.941$, $p = 0.001$) (Fig. 4(A)). Moreover, baseline SICI correlated with the CRS-R score, i.e. a higher CRS-R score was accompanied by a lower baseline SICI magnitude ($r = -0.884$, $p = 0.001$) (Fig. 4(B)). We found that the MEP amplitude modulation was higher at a higher CRS-R score ($r = 0.894$, $p = 0.001$)

(Fig. 4(C)). The overall association between baseline SICI, MEP amplitude increase following TDCS, and CRS-R score are summarized in Fig. 5. In particular, we observed that the MEP amplitude increase was paralleled by stronger baseline SICI and higher CRS-R scorings. Three patients with UWS ($n = 3, 16$, and 19) showed a clinical–electrophysiological correlation that was much higher than that shown by the other patients with UWS.

Table 2. ANOVA findings concerning TDCS over primary motor cortex. The signs in parentheses (+/−) refer to the facilitatory or inhibitory effect of the TCDS polarity employed on the parameter, measured as % of the unconditioned MEP. Nonreported data (ICF, UWS group, sham-cathodal TDCS, and T3-TPRE interval) are nonsignificant.

Time × group × priming $F_{(24,1488)}$	Priming × time HC: $F_{(12,288)}$; MCS: $F_{(12,216)}$	Time HC: $F_{(3,72)}$; MCS: $F_{(3,54)}$	Post-hoc <i>t</i> -test	
			$T1-T_{PRE}$	$T2-T_{PRE}$
MEP $F = 7.7, p < 0.001,$ $\lambda = 184, \eta^2 = 0.9$	HC $F = 21, p < 0.001,$ $\lambda = 249, \eta^2 = 0.9$	(+) c-c $F = 23, p < 0.001,$ $\lambda = 69, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.8$
		(+) c-a $F = 9.3, p < 0.001,$ $\lambda = 28, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p = 0.005$ $d = 0.6$
		(−) a-a $F = 21, p < 0.001,$ $\lambda = 249, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p = 0.008$ $d = 0.6$
		(−) a-c $F = 9.3, p < 0.001,$ $\lambda = 28, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.8$
	MCS $F = 17, p < 0.001,$ $\lambda = 208, \eta^2 = 0.9$	(+) c-c $F = 11, p < 0.001,$ $\lambda = 36, \eta^2 = 0.9$	$p < 0.001$ $d = 0.6$	$p = 0.001$ $d = 0.6$
		(+) c-a $F = 14, p < 0.001,$ $\lambda = 43, \eta^2 = 0.9$	$p = 0.002$ $d = 0.6$	$p = 0.005$ $d = 0.5$
		(+) c-a $F = 28, p < 0.001,$ $\lambda = 84, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p = 0.003$ $d = 0.7$
		(−) a-c $F = 34, p < 0.001,$ $\lambda = 104, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p = 0.005$ $d = 0.5$
SICI $F = 9.1, p < 0.001,$ $\lambda = 219, \eta^2 = 0.9$	HC $F = 16, p < 0.001,$ $\lambda = 186, \eta^2 = 0.9$	(−) a-c $F = 19, p < 0.001,$ $\lambda = 56, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p = 0.007$ $d = 0.6$
	MCS $F = 8, p < 0.001,$ $\lambda = 96, \eta^2 = 0.9$			

Finally, receiver operating characteristic (ROC) analysis showed that the diagnostic accuracy of the overall SICI strength modulation was excellent (0.96) (Fig. 6(A)), i.e. the patients with MCS showed a TDCS-induced SICI strengthening, whereas the patients with UWS did not. In contrast, MEP amplitude (Fig. 6(B)) and ICF strength (Fig. 6(C)) modulations were poorly associated with DoC category (0.63 and 0.57, respectively).

4. Discussion

To the best of our knowledge, this is the first study investigating metaplasticity in the patients

with DoC. Our experimental paradigm was able to distinguish between MCS and UWS by inducing significant changes in SICI that strongly correlated with the CRS-R scoring.

4.1. Putative neurophysiological mechanisms of TDCS-induced metaplasticity

Healthy individuals showed some modulation of primary motor and premotor-motor excitability following the different types of TDCS consistent with homeostatic mechanisms of metaplasticity, i.e. the equal polarities yielded consistent

Fig. 4. The scatterplots of the significant correlations between CRS-R and electrophysiological findings (overall TDCS-induced modulation of SICI and MEP magnitude, baseline SICI strength). $\blacktriangle$ indicate patients with UWS, $\blacklozenge$ those with MCS, $\bullet$ those with the supposed Functional Locked-In Syndrome.

facilitatory or inhibitory after-effects, whereas the opposite polarities induced diametrically opposed effects as compared to the same stand-alone polarities.^{38,47–55}

The underlying cellular and molecular mechanisms supporting these metaplasticity models are partially known. TDCS-induced metaplasticity could depend on cellular mechanisms within the pyramidal neurons⁵⁶ at least when using the same TDCS polarity (i.e. cathodal–cathodal and anodal–anodal). In fact, the measurability of TDCS after-effects depends on the paired-pulse interval tested.^{38,57,58} In addition, the paired-pulse TMS interval we used could test glutamatergic and γ -aminobutyric acidergic neurons that are different from those involved in the homeostatic plasticity.³⁸

The finding agrees with the previous experimental models highlighting the role of the inhibitory synapses concerning metaplasticity regulation.^{43,54,59–64} The discrepancy between the homogeneous and non-homogeneous TDCS polarities concerning SICI may depend on a different entrainment of the inhibitory neuronal networks surrounding pyramidal cells (probably generating late *I*-waves),⁶⁴ depending on the TDCS polarities and intervals employed.^{23,65–68} Indeed, short-lasting TDCS protocols may target *L*-type voltage-gated Ca^{2+} channels^{67,69} that regulate the magnitude of LTP/LTD strength (i.e. the high levels of postsynaptic Ca^{2+} favor LTP, whereas the low levels favor LTD; thereafter, preceding high levels of postsynaptic Ca^{2+} favor LTD, whereas low levels favor LTP).^{70,71} Moreover, some inhibitory populations can be shaped more quickly by the opposite TDCS polarities, whereas other populations may not show sufficient response depending on the smallness of threshold as compared to pyramidal neurons^{43,64} and the previous synaptic activity.³⁸ MEP amplitude showed less evident TDCS-induced changes, as compared to SICI and ICF. However, MEP is a complex measure of corticospinal excitability and is influenced by either cortical or subcortical circuits projecting into the corticospinal motor neurons.^{72–74} Moreover, subtle homeostatic changes may have an effect size that remains within the noise level of normal fluctuations in MEP amplitude.^{38,75} Further, ICF is barely changed by primary motor cortex metaplasticity protocols. Methodological factors may avoid an ICF modulation, which requires posteroanterior current flow (while inhibition can be elicited regardless of the direction of current flow).⁶³ In fact, the premotor–primary motor cortex TDCS induces rather clear ICF

Table 3. ANOVA findings concerning TDCS over premotor cortex. The signs in parentheses (+/−) refer to the facilitatory or inhibitory effect of the TDCS polarity employed on the parameter, measured as % on the unconditioned MEP. Non-reported data (UWS group, sham-cathodal TDCS, and T3-TPRE interval) are nonsignificant.

Time × group × priming $F_{(24,1488)}$	Priming × time HC: $F_{(12,288)}$; MCS: $F_{(12,216)}$	Time HC: $F_{(3,72)}$; MCS: $F_{(3,54)}$	Post-hoc t -test	
			$T1-T_{PRE}$	$T2-T_{PRE}$
MEP $F = 5.4, p < 0.001,$ $\lambda = 129, \eta^2 = 0.9$	HC $F = 10, p < 0.001,$ $\lambda = 120, \eta^2 = 0.9$	(+) c-a $F = 45, p < 0.001,$ $\lambda = 136, \eta^2 = 0.9$	$p < 0.001$ $d = 0.8$	$p < 0.001$ $d = 0.8$
		(−) a-a $F = 4.1, p = 0.009,$ $\lambda = 12, \eta^2 = 0.8$	$p < 0.001$ $d = 0.8$	$p < 0.001$ $d = 0.7$
	MCS $F = 2.6, p = 0.003,$ $\lambda = 31, \eta^2 = 0.8$	(+) c-a $F = 17, p < 0.001,$ $\lambda = 52, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.8$
		(−) a-a $F = 7.8, p < 0.001,$ $\lambda = 23, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.8$
		(+) c-a $F = 92, p < 0.001,$ $\lambda = 278, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.9$
		(−) a-a $F = 2.7, p = 0.04,$ $\lambda = 8.1, \eta^2 = 0.6$	$p < 0.001$ $d = 0.8$	$p = 0.01$ $d = 0.+$
SICI $F = 4.8, p < 0.001,$ $\lambda = 115, \eta^2 = 0.9$	HC $F = 21, p < 0.001,$ $\lambda = 248, \eta^2 = 0.9$	(−) a-a $F = 16, p < 0.001,$ $\lambda = 47, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p = 0.001$ $d = 0.7$
	MCS $F = 8, p < 0.001,$ $\lambda = 96, \eta^2 = 0.9$	(+) c-a $F = 46, p < 0.001,$ $\lambda = 138, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.9$
ICF $F = 4.5, p < 0.001,$ $\lambda = 113, \eta^2 = 0.9$	HC $F = 12, p < 0.001,$ $\lambda = 140, \eta^2 = 0.9$	(+) c-a $F = 19, p < 0.001,$ $\lambda = 60, \eta^2 = 0.9$	$p < 0.001$ $d = 0.9$	$p < 0.001$ $d = 0.9$
	MCS $F = 5.7, p < 0.001,$ $\lambda = 68, \eta^2 = 0.9$			

changes.^{76–78} Therefore, other homeostatic plasticity mechanisms might contribute to TDCS-induced metaplasticity,⁶⁴ which should be addressed in future studies.

4.2. Metaplasticity in patients with DoC

Metaplasticity was largely abnormal in patients with DoC. Indeed, both DoC groups showed a tonically high cortical excitability as compared to HC individuals, but the patients with MCS showed a greater TDCS-induced modulability of SICI (and partially of ICF and MEP) than UWS individuals

did (except patients *n.* 3, 16, and 19). In particular, the degree of metaplasticity abnormality, measured through the baseline SICI and TDCS-induced SICI modulation, was inversely correlated with the CRS-R scoring. AUC analysis confirmed that only SICI well distinguished between the DoC groups. Such data confirm the important role of the degree of functional impairment of cortical, and even thalamo-cortical, inhibitory circuitries concerning the awareness impairment estimated by the CRS-R,^{26,28,79–84} independently from the integrity of corticospinal tract (as reflected by the mild changes of MEP amplitude).^{85,86} Therefore, the assessment of the brain responses to TDCS-based

Fig. 5. Association between baseline SICI, MEP amplitude modulation following TDCS over primary motor cortex, and CRS-R scoring. Some among the patients with UWS (those in parentheses) showed electrophysiological values largely outside those shown by the other patients with UWS.

Note: UWS Unresponsive Wakefulness Syndrome, MCS Minimally Conscious State, *aa* both-anodal, *ac* anodal-cathodal, *ca* cathodal-anodal, *cc* both-cathodal.

metaplasticity protocols may indicate the amount of residual plasticity that correlates with the degree of awareness, thus differentiating patients with DoC.

Cortical disexcitability in the patients with DoC may depend on a decoupled activity of facilitatory (*N*-methyl-*D*-aspartate) and inhibitory

(γ -aminobutyric acid) interneurons following brain injury,^{87,88} where the former can facilitate the deterioration of the latter, in keeping with the severity of brain damage. This could be the reason why ICF was less modulable and so had a marginal role in distinguishing patients with DoC. It has been proposed that the drop in inhibitory circuits offers more favorable conditions for the development of restorative plastic changes that facilitate the restoration of the impaired networks.^{33,34} This cortical facilitation is a consequence of cortical deafferentation due to the thalamo-cortical projection deterioration, and is also influenced by other factors influencing those neurotransmitters that are important for metaplasticity regulation (including the threshold for post-ischemic LTP,⁸⁹ nitric oxide signaling,⁹⁰ the ignition of delayed apoptosis-like neuronal death process,⁹¹ and the release of stress hormones^{13–16}).

Cortical disexcitability in patients with DoC was further demonstrated by the lack of effects of single cathodal TDCS (in the sham protocol), which instead affected SICI in HC as formerly shown.^{92,93} At the same time, the sham protocol would rule out a possible causal effect of double TDCS protocols on cortical excitability.

Three patients with UWS (*n.* 3, 16, and 19) showed residual TDCS-induced responses that were similar to those shown in patients with MCS. However, such patients with UWS showed a CRS-R score that was the same of the other patients with UWS, who although showed nonsignificant TDCS-induced brain responses. A similar condition may suggest a diagnosis of Functional Locked-In Syndrome, which is characterized by a residual brain connectivity (suggesting awareness preservation) despite a severe behavioral impairment.^{94–97} This dissociation probably depends on a severe deterioration of sensory-motor circuitries.³⁰ However, this finding was interpreted cautiously as this TDCS after-effect was noted only in one individual.

4.3. Limitation and conclusions

One could concern that the relatively small sample size limits the significance of multiple comparisons made (as the required sample size increases linearly with the logarithm of the number of comparisons made). However, the required sample size for detecting a between-group difference between TDCS

Fig. 6. The power of SICI, MEP, and ICF modulation in differentiating patients with DoC is shown by the AUC of the ROC in panels (A)–(C), respectively.

after-effects using the typical conventions of 80% power, a two-tailed alpha of 0.05, and a putative metaplasticity effect size of approximately 0.40 (in keeping with the available literature data on TDCS in patients with DoC),^{98–105} should be of at least 35 subjects,^{106,107} which is satisfied by our sample. Further, the differences between the patients with DoC were statistically significant, as also shown by the effect size data indicating a big effect of TDCS on SICI, above all. Finally, to recruit a sufficient number of patients with chronic DoC in a single center study is challenging, due to the unfortunately high negative outcome and the thorny selection of patients based on the inclusion criteria required by the typology of the study.

We can only speculate on the influence of TDCS on thalamo-cortical connectivity in patients with DoC, given that we tested only a small slice of brain connectivity, i.e. between the premotor and motor cortices. However, the frontal lobe TDCS is known to modulate thalamo-cortical connectivity through multiple fronto-striatal connections,^{108,109} beyond the facilitatory projections from premotor to motor cortex,¹¹⁰ as suggested by the ICF increase during premotor–motor TDCS. The premotor–motor excitability was clearly deteriorated in the patients with DoC in correlation with the CRS-R score. This further confirms that a connectivity breakdown within distinct cortical areas accounts for the loss of consciousness in patients with DoC,^{111–115} and suggests that the connectivity impairment between premotor and motor cortices is significant to cause motor output deterioration, even when

a patient shows a partially preserved whole brain connectivity.^{29,30,116}

One could argue that the lack of conditioning after-effects in patients with DoC may depend on the fact that TDCS fails to recruit the SICI networks,^{117,118} rather than on a low amount of plasticity. However, the magnitude of TDCS after-effects depended on baseline SICI values; therefore, if a patient has a sufficient SICI, we may assume that the lack of response to TDCS may properly indicate a reduced plasticity, thus differentiating patients with DoC.

Given the small number of available studies dealing with TDCS in patients with DoC,^{100–105} we can only try to quantify the relevance of our method in comparison with the other state-of-the-art methods. Our data indicate that metaplasticity is valuable in differentiating patients with DoC. Larger cohort studies comparing different sites of stimulation (since some patients may benefit from TDCS targeting different cortical areas, based on their brain lesions) are therefore needed to confirm the possible superiority of one specific approach.

In conclusion, our promising data suggest that the assessment of metaplasticity may quantify the residual plasticity properties in patients with DoC, which correlate with the degree of awareness. Therefore, metaplasticity assessment could be used to corroborate (or not) clinical DoC diagnosis, potentially reducing the still high misdiagnosis rate.³² Moreover, we have to acknowledge the relevance of metaplasticity in Bio-Inspired Machine Learning, which could usefully be employed in the management

of patients with DoC.^{119–121} Finally, metaplasticity assessment in patients with DoC may identify the subjects who could benefit from neuromodulation protocols, and help the clinicians implementing more personalized neurorehabilitative training for such a very frail and vulnerable group of patients.

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