Restorative Neurology and Neuroscience 33 (2015) 447–460
DOI 10.3233/RNN-150525
IOS Press

Visuo-motor integration in unresponsive wakefulness syndrome: A piece of the puzzle towards consciousness detection?

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Abstract.
Purpose: The unresponsive wakefulness syndrome (UWS) is characterized by either a profound unawareness or an impairment of large-scale cortico/subcortical connectivity. Nevertheless, some individuals with UWS could show residual markers of consciousness and cognition. In this study, we applied an electrophysiological approach aimed to identify the residual visuomotor connectivity patterns that are thought to be linked to awareness, in patients with chronic disorder of consciousness (DOC).
Methods: We measured some markers of visuomotor and premotor-motor integration in 14 patients affected by DOC, before and after the application of transcranial direct current stimulation, delivered over the dorsolateral prefrontal cortex and the parieto-occipital area, paired to transorbital alterning current stimulation.
Results: Our protocol induced a potentiation of the electrophysiological markers of visuomotor and premotor-motor connectivity, paired to a clinical improvement, in all of the patients with minimally conscious state and in one individual affected by UWS.
Conclusions: Our protocol could be a promising approach to potentiate the functional connectivity within large-scale visuomotor networks, thus allowing identifying the patients suffering from a functional locked-in syndrome (i.e. individuals showing an extreme behavioral motor dysfunction although with somehow preserved cognitive functions that can be identified only through para-clinical tests) within individuals with UWS.

Keywords: DLPFC, functional connectivity, MCS, parieto-occipital area, tDCS, UWS, visuomotor integration

1. Introduction

The clinical detection of awareness signs, including purposeful behavioral responsiveness to stimuli, has a pivotal role in the differential diagnosis of chronic disorders of consciousness (DOC). In fact, the lack of awareness characterizes the unresponsive wakefulness syndrome (UWS) (previously named vegetative state - VS), whereas patients with minimally conscious state (MCS) show "inconsistent but clearly discernible behavioral evidence of consciousness" (Giacino et al., 2002; Laureys et al., 2010a). Notably, awareness impairment and, thus, the limitation of behavioral responsiveness are proportionally related to the level of connectivity disruption within a wide cortical/subcortical neuromatrix that supports consciousness generation and maintenance, besides the arousal system (Laureys et al., 2010b). Nevertheless, the patients suffering from a functional locked-in syndrome (fLIS) show an extreme behavioral motor dysfunction although with a partial preservation of higher cognitive functions and cerebral connectivity, as advanced neurophysiological and neuroimaging approaches have

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ISSN 0922-6028/15/$35.00

shown (Bruno et al., 2011). Such condition may arise from lesions at one or more levels of the sensory-motor system. On the other hand, patients with classic (i.e., quadriplegia and anartria, with eye-coded communication) and total LIS (i.e., a rare syndrome characterized by complete immobility including eye movements) typically suffer from a brainstem injury and show very limited signs of awareness due to profound sensory and motor deficits, although with preserved self-awareness and cognitive capacities, besides a normal brain network connectivity (Haig et al., 1987; Laureys et al., 2005). Indeed, it is extremely difficult to properly assess awareness in such patients, since either their movements may be minimal or inconsistent, or no cognitive output could be possible (Giacino and Zasler, 1995). In fact, fLIS, total LIS, and patients suffering from UWS are behaviorally indistinguishable, and it is possible to reach the differential diagnosis only through paraclinical approaches, including functional neuroimaging and advanced neurophysiological paradigms (Bruno et al., 2011).

Hence, a covert cognition and a residual complex cerebral connectivity characterize patients affected by fLIS and total LIS, whereas individuals with UWS lack of such markers. Nevertheless, the study of a sensory-motor system that is unable to contribute to the generation of purposeful behaviors, is challenging in patients with DOC, and thus the misdiagnosis rate is still high (Bekinschtein and Manes, 2008).

To this end, the study of large-scale sensory-motor integration processes supporting awareness may be useful in an attempt to differentiate patients with fLIS (Kotchoubey et al., 2013). In particular, the visuomotor integration (VMI) assessment seems a promising approach, as suggested by its clinical usefulness in terms of differential diagnosis and prognosis (Troiano et al., 2012; Hildebrandt et al., 2007). Moreover, VMI enrolls different and complex cortical-subcortical networks that are related to visual processes at conscious level (Grossberg, 2003; Humphreys et al., 1997). On the other hand, the first-level VMI processes (i.e. the paired activation of primary motor and primary visual cortices) do not correlate with awareness preservation (reflecting instead alerting functions, motor attention and preparation) (Katsuki and Constantinidis, 2012; Monti et al., 2013).

Several works have shown the possibility to bring to light the above mentioned complex integrative processes by means of non-invasive neurostimulation techniques, including the repetitive transcranial magnetic stimulation (rTMS) and the transcranial direct current stimulation (tDCS). Such approaches could boost neural plasticity by means of long-term potentiation or depression-like mechanisms (LTP or LTD) (Ziemann et al., 2008) within the primary motor cortex (M1) (Rioult-Pedotti et al., 2000; Sanesand Donoghue, 2000), the sensory-motor areas (Stefan et al., 2000; Wolters et al., 2003), and the pain-matrix (Garcia-Larrea and Peyron, 2013; Suppa et al., 2013; Naro et al., 2015b). In addition, such paradigms do not necessarily require a substantial patient's cooperation. Interestingly, the assessment and the modulation of VMI through non-invasive neuromodulation could be helpful in the differential diagnosis of UWS and fLIS. Indeed, we may argue that patients with UWS who show an improvement of the cortical functional connectivity and the visuomotor output after a proper neuromodulation approach should be no longer considered as UWS, but fLIS. To this end, we assessed the large-scale visuo-premotor-motor functional connectivity in a clinically defined UWS sample and in a control group of MCS and healthy subjects (HC), before and after the administration of a paired associative stimulation protocol consisting of tDCS over dorsolateral prefrontal cortex (DLPFC) and parieto-occipital areas (POA), and trans-orbital alterning current stimulation (tACS). More in detail, we measured some parameters of cortical excitability and connectivity, by means of single- and dual-site TMS, and the visual-stimuli event-related potentials. Since a standard visual pathway stimulation is extremely challenging in patients with DOC, owing to the low and inconsistent cooperation, we chose a tACS approach capable to evoke visual potentials regardless patient's cooperation. We stimulated the DLPFC and the POA by means of the dual-site tDCS, because of their important role in the stimulus-driven (bottom-up) and the purpose-driven (top-down) neural processes (Grossberg, 2003; Humphreys et al., 1997). More in detail, the POA's intrinsic circuits constitute the common origin of three distinct pathways: i) the parieto-prefrontal, involved in top-down control of eye movements and in spatial working memory; ii) the parieto-premotor, mediating ocular, reaching, and grasping movements; and iii) the parieto-temporal, underlying the complex spatial processing required for navigating through the environment.

Since voluntary eye-movements are an important clinical marker of the level of awareness, we chose such double-site tDCS approach.

Table 1
The Clinical and demographic characteristics of the whole sample. We reported the individual CRS-R mean values ± sd (the CRS-R was daily administered for 30 consecutive days before the protocol enrollment). We marked in bold the patients who showed a visuo-motor improvement after the real_protocol application

DOC	gender	etiology	age	BI	MRI	CRS-R						
						total	A	V	M	OM	C	Ar
MCS	F	A	72	6	WMH	18 ± 0.7	4 ± 1.4	4 ± 0.6	5 ± 1.9	1 ± 1.8	1.8 ± 0.9	3 ± 1.3
	M	T	51	18	WMH, ^{R}BG_h	15 ± 1.6	3 ± 1.5	3 ± 0.9	4 ± 1.7	1 ± 1.2	0.6 ± 0.3	3 ± 0.8
	F	A	66	9	WMH	12 ± 1.7	1 ± 0.9	3 ± 1.2	2 ± 0.8	2 ± 0.7	1.6 ± 0.9	3 ± 0.9
	F	**T**	**70**	**22**	**^{L}Fb_h**	**15 ± 0.6**	**3 ± 1.6**	**2 ± 0.3**	**5 ± 0.5**	**1 ± 0.6**	**0.6 ± 0.2**	**3 ± 1.9**
	M	**T**	**33**	**8**	**multiple h**	**13 ± 0.8**	**2 ± 1**	**2 ± 0.7**	**3 ± 0.5**	**2 ± 1.2**	**1.9 ± 0.5**	**3 ± 1.4**
	F	A	41	15	WMH	12 ± 1	1 ± 0.5	1 ± 0.6	3 ± 0.5	2 ± 0.3	0.6 ± 0.4	3 ± 1.9
	M	T	35	16	WMH, ^{R}BG_h	11 ± 0.2	1 ± 1.8	1 ± 1.7	3 ± 0.7	2 ± 1.8	1.7 ± 0.6	3 ± 0.1
mean±sd			*53 ± 17*	*13 ± 6*		*14 ± 0.9*	*2 ± 1.2*	*2 ± 0.9*	*4 ± 0.9*	*1.6 ± 1.1*	*1 ± 1.3*	*3 ± 1.1*
UWS	M	A	53	8	WMH	5 ± 0.4	1 ± 1.1	1 ± 1.2	1 ± 1.1	1 ± 0.6	0.4 ± 0.1	1 ± 1.5
	F	T	26	3	DAI, SAH	4 ± 1.4	1 ± 0.7	1 ± 0.8	1 ± 1.5	0 ± 1.4	1.3 ± 1	1 ± 0.6
	F	**T**	**66**	**8**	**^{R}FP_h**	**7 ± 1**	**0 ± 1.2**	**2 ± 1.5**	**2 ± 1.4**	**1 ± 1.4**	**1 ± 1**	**1 ± 2**
	F	A	62	11	WMH	6 ± 1	1 ± 1.3	1 ± 1.2	2 ± 0.6	0 ± 0.3	0.8 ± 0.9	2 ± 0.4
	M	T	61	9	SAH	4 ± 0.7	1 ± 1	1 ± 0.2	1 ± 0.2	0 ± 1.2	1.1 ± 0.2	1 ± 1.6
	M	A	69	11	WMH	7 ± 1.3	1 ± 0.1	1 ± 0.7	2 ± 0.7	1 ± 1.1	1.4 ± 0.6	2 ± 0.9
	F	T	74	12	DAI, SAH	6 ± 0.3	1 ± 1.4	2 ± 1.4	1 ± 1.4	0 ± 0.7	0.4 ± 0.6	2 ± 1.4
mean±sd			*59 ± 16*	*9 ± 3*		*6 ± 1*	*0.9 ± 0.4*	*1.3 ± 0.5*	*1.5 ± 0.5*	*0.4 ± 0.5*	*0.9 ± 0.6*	*1.6 ± 0.5*

Etiology: A, post-anoxic, T, post-traumatic brain injury; BI: brain injury onset in months; age in years; MRI: structural patterns including WMH (white matter hyper-intensity), _h (hemorrhagic lesion), ^{R}FP (right fronto-polar), ^{R}BG (basal ganglia), ^{L}Fb (left fronto-basal), SAH (sub-arachnoid hemorrhage), DAI (diffuse axonal injury); CRS-R: Coma Recovery Scale-revised including auditory (A), visual (V), motor (M), oro-motor (OM), communication domain (C), and arousal induction (Ar); sd: standard deviation.

We hypothesized that our experimental protocol could improve the motor area excitability, the visuo-prefrontal functional connectivity, and the visuomotor output, allowing bringing to light covert signs of awareness in patients who were clinically defined UWS.

2. Methods

2.1. Subjects

Of the 32 subjects suffering from chronic DOC attending the Neurorehabilitative Unit of the IRCCS Centro Neurolesi "Bonino-Pulejo" (Messina, Italy), we enrolled 14 patients who met the criteria for VS/MCS diagnosis (The Multi-Society Task Force on PVS, 1994; Giacino et al., 2002), and the following exclusion criteria: a DOC condition lasting less than 3 months after the brain injury; other severe neurological or systemic diseases; critical conditions (i.e. inability to breathe independently, hemodynamic instability); cortical excitability-modifying drugs assumption beyond L-DOPA and baclofen; presence of epileptic history, pace-maker, aneurysms clips, neurostimulator, brain/subdural electrodes or other electromechanical devices; presence of electroencephalographic (EEG) suppression-burst pattern; absence of visual evoked potential (VEP). In addition, we included in the study 7 HC (4 females and 3 males, mean age 55.3 ± 5.8 years) as control group.

We reported the clinic-demographic characteristics in Table 1. DOC etiology consisted of a post-anoxic or a post-traumatic brain damage.The neurological examination mainly showed a pattern of spastic tetraparesis. EEG examination evidenced a continuous slowing in theta and/or delta frequency ranges. Our Research Institute Ethics Committee approved the present study and either the HC or the legal guardian of each patient gave their written informed consent.

2.2. Clinical assessment

Two neurologists skilled in DOC diagnosis independently evaluated the patients through the JFK CRS-R. This scale is a reliable and standardized tool, which integrates neuropsychological and clinical assessment, and includes the current diagnostic criteria for coma, VS, and MCS, allowing the clinician to assign the patient to the most appropriate diagnostic category. Hence, the CRS-R represents an appropriate approach for characterizing the level of consciousness and

for monitoring the neurobehavioral function recovery (Gerrard et al., 2014). The CRS-R was daily administered for 30 consecutive days, at different times, in order to steadily establish the level of consciousness impairment.

2.3. Conditioning protocols

Each participant underwent four different protocols: i) a real (real_tDCS+real_tACS); ii) a sham (sham_tDCS+sham_tACS); iii) a tDCS_alone (real_tDCS + sham_tACS); and iv) a tACS_alone (real_tACS+ sham_tDCS). We administered the protocols in a random scheme (i, ii, iii, and iv) and in different sessions, at one-day of interval. Participants and experimenters who analyzed data were blinded on scheme procedure.

For the real_tDCS, we used a battery-driven stimulator (Brain Stim, E.M.S., Bologna, Italy) with a couple of conductive-rubber electrodes, placed in saline-soaked sponges (active electrode 5×5 cm) over F3 (DLPFC) and PO5 (POA) (according to the 10/20 International System). The current stimulation ramped up/down during the first/last 30 sec of stimulation, at 1 mA of intensity. Current density was always below the safety limit of 52 μA/cm^2. The device kept the impedance below 10 kΩ (Miranda et al., 2006; Bikson et al., 2010; Nitsche et al., 2003).

For the real_tACS, we extra-ocularly applied 4 electrodes (sintered Ag/AgCl ring electrodes) to both eyelids of the left eye (while the eye was kept closed), cabled to a battery-driven stimulator (BrainStim, E.M.S., Bologna, Italy) (Sabel et al., 2011). FPz served as reference electrode. At first, we determined the phosphene threshold in our HC sample, applying bursts of 15 pulses (10 ± 2 ms at 20 ± 5 Hz; the values ranged randomly in order to avoid habituation phenomena) and increasing the current intensity by steps of 10 μ A per second (starting from zero). We set the stimulation frequency in HC, increasing the frequency by steps of 1–5 Hz per second, from the α-range (the minimum stimulation frequency applicable) to the flicker-fusion frequency (i.e. the maximal temporal resolution frequency at which the intermittent tACS stimulus appeared as steady to the average of the HC), at an intensity of $\leq$0.8 mA.The mean values of these parameters were applied in each participant affected by DOC.

Concerning the real_protocol, we delivered 150 visual stimuli during each tDCS protocol, at the same intensity used in VEP elicitation, at 0.25 Hz (i.e., 300 stimuli in 20 min). For the sham_tDCS, we switched-off the stimulator after 30 sec, whereas the sham_tACS consisted of 50 stimuli (i.e. at 0.08 Hz).

2.4. Single-pulse, paired-pulse and dual-site TMS measures

We used a high-power Magstim200^2 Stimulator (Magstim, Whitland, Dyfed, UK) in order to elicit the motor evoked potential (MEP) through magnetic monophasic stimuli. We held the coil tangentially to the scalp with the handle pointing backwards and laterally, at a 45° angle from the sagittal plane, approximately perpendicular to the central sulcus of the left hemisphere, on the optimal scalp site to get the wider MEP amplitude from the relaxed right abductor pollicis brevis muscle (APB) (motor hot-spot). The rise time of the magnetic monophasic stimulus was $\sim$100 μs with a to-zero of $\sim$800 μs. The current flowed in handle direction during the rise-time of the magnetic field, thus with a posterior-anterior direction. We preliminarily evaluated the resting motor threshold (RMT), defined as the smallest magnetic stimulus intensity able to evoke a peak-to-peak MEP of 50 uV in resting right APB, in at least five-out-ten consecutive tracks (Rossini et al., 1994). Then, we applied an intensity of stimulation of 120% of RMT (test stimulus). We applied Ag-AgCl surface electrodes to the right APB, using a classic muscle belly-tendon montage, for recording EMG activity. A Digitimer D360 Amplifier (Digitimer Ltd., Welwyn Garden City, Herts, UK) amplified, filtered (32 Hz-1 KHz), and stored the signals (at 10KHz on a personal computer for off-line analysis) (Signal Software, Cambridge Electronic Design, Cambridge, UK). During the experiments, we continuously monitored the EMG activity through visual (oscilloscope) and auditory (speakers) feedback, to ensure the complete muscle relaxation.

Concerning the short-latency intracortical facilitation (SICF), we delivered two juxta-RMT stimuli (being the former the conditioning stimulus, the latter the test one) at an interstimulus interval of 2.5 ms (Tokimura et al., 1996), whereas we applied two suprathreshold stimuli (at 120% RMT) at an interstimulus interval of 150 ms for the long-latency intracortical inhibition (LICI) (Valls-Sole et al., 1992). We measured the mean amplitude of the conditioned MEP as percentage of the amplitude of the unconditioned MEP (test), which was taken as a measure of cortical excitability. We registered 15 unconditioned MEPs, 10 LICI, and 10 SICF, randomly intermingled in a single

trial, at a frequency of 0.25 Hz (test duration ∼9 min). All data are given as mean or percentage difference in comparison to baseline values ± standard error (se).

Concerning premotor-motor interaction, we measured the effects on MEP amplitude of a dual-site TMS approach (using two figure-of-eight monophasic coils) on PMd_{right}-$M1_{left}$ and PMv_{left}-$M1_{left}$ (interstimulus interval of 7 ms, conditioning stimulus at 70% RMT on the premotor area, test stimulus over M1 at 120% RMT), SMA-$M1_{left}$ (CS at an interstimulus interval of 6 ms, conditioning stimulus at 3 cm anteriorly to Cz -preSMA- at 70% RMT, test stimulus over M1 at 120% RMT), and PPC_{left}-$M1_{left}$ (conditioning stimulus at an interstimulus interval of 10 ms, at 90% RMT, test stimulus over M1 at 120% RMT) (Civardi et al., 2001; Koch et al., 2007a,b; Davare et al., 2008). We registered10 conditioned MEPs for each premotor-motor interaction in different trials, intermingled with 10 unconditioned MEPs, delivered at a frequency of 0.25 Hz (test duration ∼12 min). We measured the mean amplitude of the conditioned MEP as percentage of the amplitude of the unconditioned (test) MEP, which was taken as a measure of premotor-motor excitability. All data are given as mean or percentage difference in comparison to baseline values ± se.

2.5. VEP, VMI, and ERP

Since a standard VEP assessment in patients with DOC is extremely challenging owing to the low and inconsistent cooperation, we chose a tACS approach in order to elicit VEP (Gall et al., 2010, 2011). We delivered 100 visual stimuli over left eye, while kept closed, with the same characteristics used for tACS, at a frequency of 1 Hz (test duration ∼1.6 min). We recorded the EEG activity from 3 scalp sites, using Ag/AgCl electrodes positioned over the mid-occipital (MO, 5 cm above nasion), left lateral occipital (LLO, 5 cm lateral to MO), and left temporal area (LT, 5 cm lateral to LLO) (Suppa et al., 2015). An electrode over the mid-frontal position (12 cm above nasion) served as reference, whereas one over right mastoid as ground. Impedance was ≤5 kΩ. We amplified and acquired the signals at 5 kHz through a 1401plus AD laboratory interface (Cambridge Electronic Design, Cambridge, UK), registered and filtered (0.5–70 Hz+50 Hz notch) through a Digitimer D360 (Digitimer Ltd, Welwyn Garden City, UK), and stored on a personal computer for off-line analysis (Signal software; Cambridge Electronic Design, UK). We registered a biphasic evoked component (N75-P100) from MO in HC, which was similar to standard pattern-reversal VEP.

In a different VEP session, we applied a tACS protocol in analogy to an odd-ball paradigm (Machado et al., 2014), in which we randomly shifted the stimulation frequency and the intensity of ± 25% in an 80:20 frequent:infrequent ratio. Each participant underwent a block of 500 trials (test duration ∼8 min), in which there was a 95% chance of 1–4 infrequent stimuli preceding a frequent one and a 5% chance of 5–7 infrequent preceding a frequent one. Hence, we were able to register an ERP from LLO and LT regardless participant's cooperation. For ERP analysis, we filtered at 0.3/25 Hz+notch (50 Hz), epoched (ranging from −100 ms by visual stimulus onset to 600 ms after) after visual and independent component analysis inspection for artifact removal, and averaged EEG signals for each channel. Then, we measured the amplitude of the registered ERP.

We assessed the VMI by means of paired visual stimuli (conditioning stimulus through left-eye tACS) and MEP (test stimulus by means of single-pulse TMS over $M1_{left}$). We chose two individually-adapted interstimulus intervals that were clearly linked to inhibitory (VEP latency +40 ms) or facilitatory (VEP latency +100 ms) effects on MEP amplitude in healthy individuals (Suppa et al., 2015). We registered 15 unconditioned MEP intermingled with 15 VEP-MEP interactions for each interstimulus intervals in a single trial, delivered at a frequency of 0.2 Hz (test duration ∼9 min). We measured the mean amplitude of the conditioned MEP as percentage of the amplitude of the unconditioned (test) MEP, which was taken as a measure of VMI strength. All the data are given as mean or percentage difference in comparison to baseline values ± se.

2.6. Study design

HC sat in a comfortable armchair in a darkened room, whereas we carried-out the patients' experimental procedure at bedside, in a darkened environment. HC and patients had their right eye covered by an eye-patch. We applied a real electrophysiological protocol, a sham, a tDCS_alone, and a tACS_alone protocol as control experiments, in each group. The protocols were delivered at one-day of interval, in a random delivery scheme (i, ii, iii, and iv). Before (T_{PRE}) and after the end of each conditioning protocol (immediately, T_0, 30-min, T_{30}, and 60-min, T_{60}), we assessed

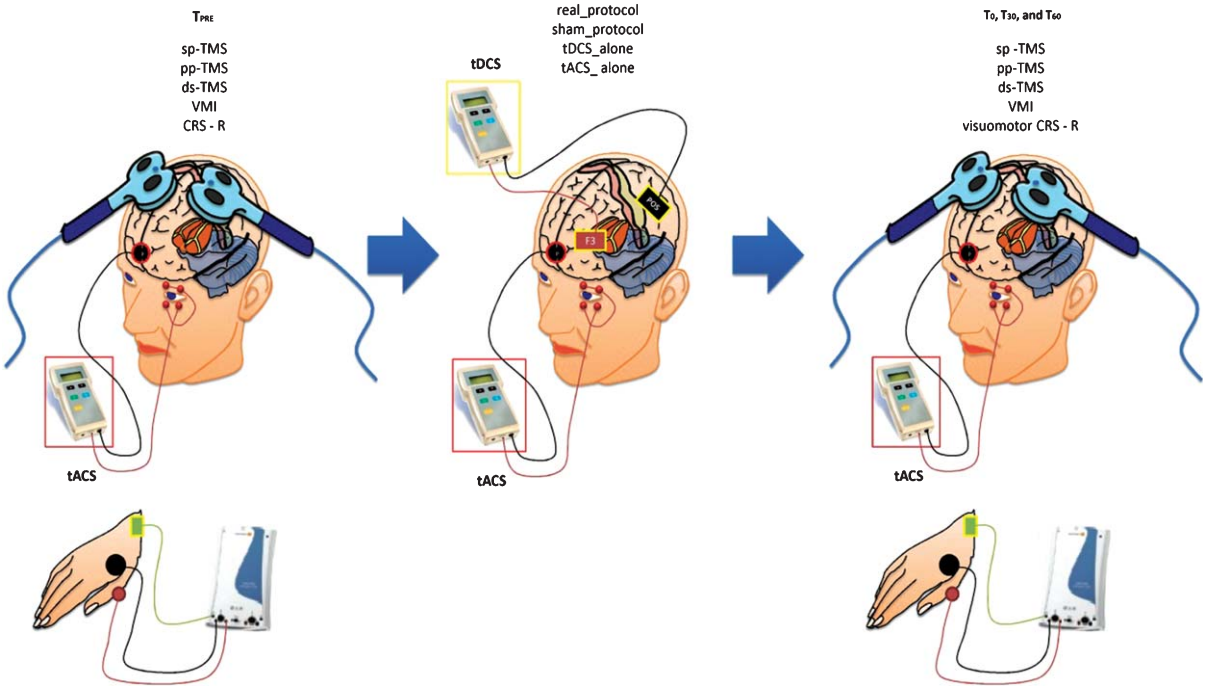

Fig. 1. Summarizes the experimental design. We carried the clinical (CRS-R) and the electrophysiological measurements (single pulse –sp-, paired-pulse –pp-, dual-site –ds- TMS, and visuomotor integration -VMI) before (T_{PRE}) and after (T_0, T_{30}, and T_{60}) each conditioning protocol: real_protocol, sham_protocol, tDCS_alone, and tACS_alone.

the peak-to-peak MEP amplitude from the left M1, the intracortical circuit excitability assessed through paired-pulse TMS over the left M1 (SICF and LICI), the premotor-motor interactions (PPC-M1, SMA-M1, PMd-M1, PMv-M1), and the VMI (combining tACS and TMS pulses over M1 at specific interstimulus intervals). Moreover, we measured the VEP and ERP parameters elicited by tACS. We individually adapted each site of TMS/tDCS stimulation and recording according to a recently performed brain MRI of each participant. We assessed the clinical effects of our protocols in patients with DOC through the JFK CRS-R. Figure 1 summarizes the experimental design.

2.7. *Data acquisition and statistical analysis*

We performed the baseline clinical and electrophysiological comparisons through unpaired *t*-tests. We evaluated the effects of the conditioning protocols on each dependent variable (visuomotor CRS-R, RMT, MEP, LICI, SICF, PMd-M1, PMv-M1, SMA-M1, PPC-M1, $VMI_{40\ ms}$, $VMI_{100\ ms}$, VEP, ERP_{LT}, and ERP_{LLO}) in separated three-way repeated-measure analyses of variance (rmANOVAs), implying *time* (four levels: T_{PRE}, T_0, T_{30}, and T_{60}) and *protocol* (four levels: real_protocol, sham_protocol, tDCS_alone, and tACS_alone) as within-subject factors, and *group* (three levels: MCS, UWS, and HC) as between-subject factor. The Greenhouse-Geisser method was used if necessary to correct for non-sphericity. Conditional on a significant F-value, we performed *post-hoc t*-tests (Bonferroni) to explore the strength of main effects and the patterns of interaction between the experimental factors. All statistical tests were applied two-tailed. A significant *p*-value was <0.05. All data are given as means or percent changes $\pm$ se. We calculated a Fisher Z-transformation in order to assess an eventual correlation among clinical, demographic, and electrophysiological parameters.

3. Results

We did not observe any effects in patients and HC, either during or after the entire experimental procedure.

Table 2

The comparison among electrophysiological parameters in HC, MCS and individuals with UWS. We found significant differences between MCS and UWS ($p_{MCS/UWS}$), and DOC and HC (*) (each *unpaired t*-tests, $p < 0.05$, except for RMT%, MEP amplitude, VEP amplitude and latency). UWS patients did not show ERPs, with a global impairment of cortical excitability, connectivity, and VEP latency and amplitude. MCS displayed an increase of cortical excitability (increased VMI_{100}, SICF, PMv, and PPC; reduced VMI_{40}, PMd, SMA, and LICI), a partially preserved premotor-motor and VMI connectivity, and the presence of ERPs limited to LO electrode. Some MCS/UWS differences approached the statistical significance (labeled as $NS^{unpaired\ t-test}$). Data are reported as mean ± se

	Measures	HC	MCS	UWS	$P_{MCS/UWS}$
sp	RMT (%)	55 ± 3	58 ± 2	60 ± 9	NS
	MEP (mV)	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	NS
ds	PMv-M1 (%)	125 ± 9*	142 ± 6	105 ± 7	<0.001
	PMd-M1 (%)	51 ± 7*	92 ± 7	105 ± 9	$NS^{0.06}$
	SMA (%)	53 ± 9*	94 ± 6	106 ± 8	$NS^{0.07}$
	PPC-M1 (%)	123 ± 11*	148 ± 6	105 ± 7	<0.001
pp	LICI (%)	60 ± 7*	94 ± 13	107 ± 5	NS
	SICF (%)	129 ± 9*	144 ± 13	167 ± 5	$NS^{0.06}$
	VMI_{40} (%)	51 ± 8*	79 ± 6	97 ± 9	<0.001
	VMI_{100} (%)	112 ± 5*	122 ± 6	108 ± 9	0.01
tACS	VEP latency (ms)	102 ± 7	115 ± 2	121 ± 3	NS
	VEP amplitude (μV)	19 ± 3	12 ± 2	8 ± 1	NS
	ERP_{LLO} latency (ms)	303 ± 10*	340 ± 15	Absent	<0.001
	ERP_{LLO} amplitude (μV)	17 ± 8*	6 ± 4	Absent	0.002
	ERP_{LT} latency (ms)	331 ± 12*	Absent	Absent	NS
	ERP_{LT} amplitude (μV)	10 ± 4*	Absent	Absent	NS

sp: single pulse TMS; ds: dual-site TMS; pp: paired-pulse TMS.

Table 3

We observed significant real_tDCS after-effects in HC and MCS patients, whereas UWS did not show any significant after-effect at group level. The tDCS_alone induced after-effects that were similar to the real_protocol but non-significant and limited to the MEP amplitude increase and the PPC-M1 potentiation. The tACS_alone and the sham_protocol were totally ineffective

	timeXgroupXprotocol interaction $F_{(18,324)}$, *p*		real_protocol	
			HC $t_{(1,6)}$, *p*	MCS $t_{(1,6)}$, *p*
MEP amplitude	3.0 <0.001	T_0	3.2, 0.02	3, 0.01
		T_{30}	2.7, 0.03	NS
		T_{60}	NS	
ERP_{LLO} amplitude	3.2, <0.001	T_0	6.1, <0.001	2.5, 0.04
		T_{30}	6, <0.001	NS
		T_{60}	NS	
ERP_{LT} amplitude	NS	T_0	6, <0.001	NS
		T_{30}	5.1, 0.005	NS
		T_{60}	NS	
PMd-M1 %	1.7, 0.04	T_0	6.4, <0.001	2.3, 0.04
		T_{30}	3.2, 0.02	NS
		T_{60}	NS	
PPC-M1 %	3.8, <0.001	T_0	3.2, 0.02	2.3, 0.04
		T_{30}	2.4, 0.04	NS
		T_{60}	NS	
SICF %	1.8, 0.02	T_0	2.9, 0.01	2.8, 0.01
		T_{30}	2.7, 0.03	NS
		T_{60}	NS	
VMI_{40} %	7.9, <0.001	T_0	9.1, <0.001	3.2, 0.02
		T_{30}	6.3, 0.003	NS
		T_{60}	NS	
VMI_{100} %	5.4, <0.001	T_0	3.8, 0.003	3.2, 0.02
		T_{30}	3.2, 0.02	NS
		T_{60}	NS	
SMA-M1 %	2.6, <0.001	T_0	2.8, 0.01	2.8, 0.01
		T_{30}	2.7, 0.03	NS
		T_{60}	NS	

3.1. DOC/HC clinical and electrophysiological differences at baseline

We showed the baseline CRS-R total scores in Table 1 (MCS-UWS comparison $p = 0.003$). There were no differences concerning either DOC etiology or the demographic characteristics. We resumed in Table 2 the baseline DOC and HC electrophysiological differences. RMT, MEP amplitude, VEP amplitude and latency were similar in the three groups, whereas paired-pulse TMS, ERP, and VMI measures were significantly different concerning either DOC-HC ($p < 0.05$ for each MCS-HC and UWS-HC comparison, by means of *unpaired t*-tests) or MCS-UWS comparison (Table 2). Indeed, the patients suffering from DOC showed an increased facilitatory (SICF) and a strongly reduced inhibitory tone (SICI), a clear alteration of cortico-cortical interactions, with a prevalence of facilitatory connections, increased latency and reduced amplitudes of VEPs and visual-ERPs (although HC showed ERPs from both LLO and LT electrodes, and the MCS only from LLO electrodes, whereas UWS did not show any ERP). Each parameter was more impaired in MCS than UWS.

3.2. Conditioning protocol's effects on clinical assessment

Although rmANOVA did not show any significant *timeXgroupXprotocol* interaction, we observed an increase of one point at the CRS-R visuomotor sub-item at T_0 in the two patients with MCS (n. 4 and 5) and in one UWS (n. 3), only after the real_protocol. Indeed, such patients with MCS upgraded from "fixation" (2 points at the CRS-R visuomotor sub-item) to "following with eyes an object" (3 points), whereas the UWS from "visual startle" (1 point) to "visual fixation" (2 points). Such effects were short-lasting, since they were not detectable at either T_{30} or T_{60}.

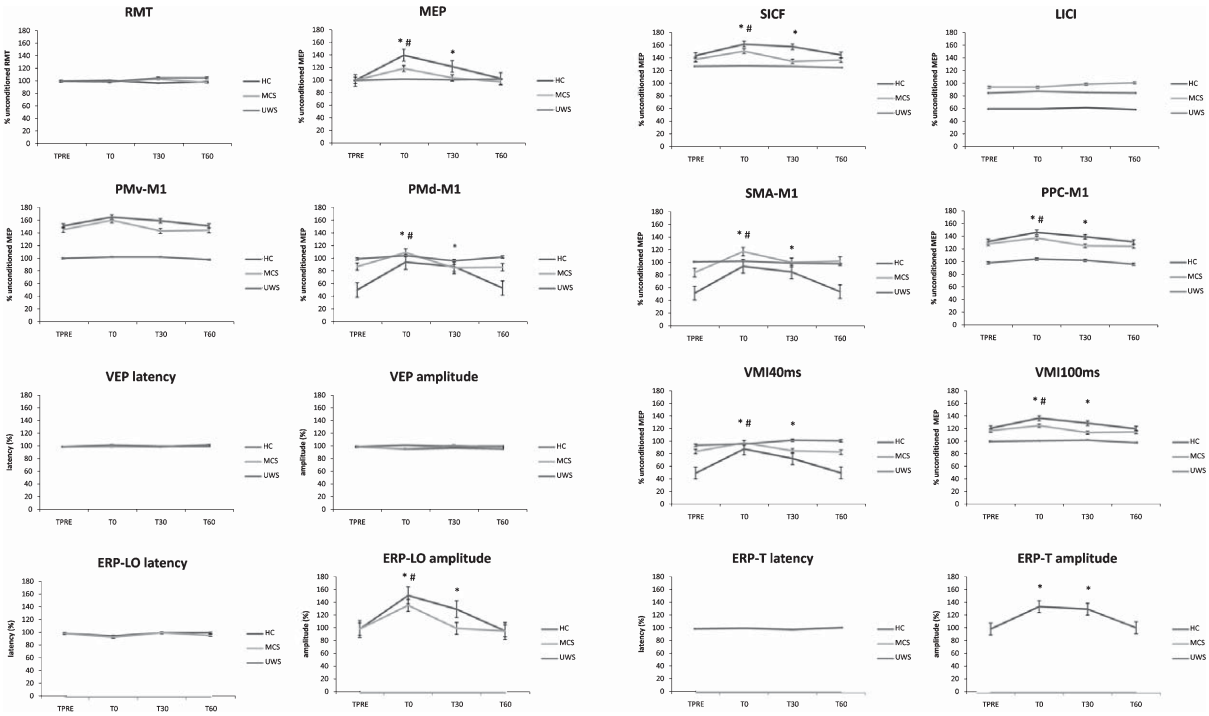

Fig. 2. The electrophysiological after-effects following the real_protocol (the ineffective protocols are not shown), which were significant up to T_{30} for HC (*), and to T_0 for MCS (#). UWS patients did not show any significant after-effects (except for n. 3). Indeed, UWS showed before and after the real_protocol a wide impairment of cortical excitability and connectivity, whereas MCS showed an amelioration of such parameters. Values are expressed as percent of the unconditioned one. Error bars refer to standard error.

3.3. Conditioning protocol electrophysiological effects

We summarized the statistical analysis data in Table 3. RMT, LICI, ERP_{LLO} and ERP_{LT} latency, PMv-M1, and VEP latency and amplitude did not significantly vary after each conditioning protocol. Concerning MEP, ERP_{LLO} amplitude, PMd-M1, and SICF, we observed a significant amplitude increase at T_0 and T_{30} in HC (the electrophysiological values reverted at T_{60} at the baseline ones), and in MCS at T_0 (the electrophysiological values reverted at T_{30} and T_{60} at baseline ones), only after the real_protocol, whereas none of the patients with UWS, but one (n.3), showed such effects (Fig. 2). PPC-M1, VMI_{40ms}, and VMI_{100ms} after-effects following real_protocol were particularly evident either in HC (up to T_{30}) or in MCS (at T_0), but not in UWS, with the exception of the patient n. 3. The tDCS_alone protocol induced after-effects that were similar to real_protocol, although non-significant and limited to MEP amplitude increase and PPC-M1 facilitation. Moreover, there were no effects to be referred to visuomotor processes. Either the tACS_alone or the sham_protocol were totally ineffective. Figure 2 shows the percentual changes of the electrophysiological parameters. Concerning the UWS subject n. 3, we found MCS-like real_protocol after-effects at T_0, which were $\sim$14% lower in strength than the mean values of patients with MCS. Moreover, we identified a small ERP_{LLO} only at T_0.

3.4. Correlations

We assessed the correlations between the amounts of electrophysiological T_{PRE}/T_0 changes in HC group, in an attempt to better understand the direction of the effects induced by the real_protocol. Interestingly, the VMI% increase was related to the PMd-M1% ($Z = 3.5$, $p = 0.001$) and the SMA-M1% increase ($Z = 2$, $p = 0.04$). Moreover, we observed a correlation between the PPC-M1 facilitation and the ERP_{LT}-amplitude increase ($Z = 3$, $p = 0.006$).

4. Discussion

Recent neurophysiological and functional neuroimaging studies have suggested that the lack of behavioral responsiveness in patients affected by DOC does not necessarily imply unawareness, since a patient could not show purposeful behavior owing to motor-output or cognitive deterioration rather than connectivity impairment (Bruno et al., 2011; Formisano et al., 2013). Interestingly, some residual patterns of cortical connectivity within visuomotor areas have been previously shown in some individuals with UWS (Owen et al., 2006; Monti et al., 2010, 2013).

For the first time ever, we assessed the presence of residual large-scale visuomotor functional connectivity in a DOC sample by means of an electrophysiological approach consisting of paired tDCS and tACS. Only the real_protocol induced a potentiation of the M1 excitability (MEP amplitude and SICF increase), the premotor-motor connectivity (increased facilitatory and decreased inhibitory connections), and the visual basic cognitive process (ERP_{LT} increase in HC, and ERP_{LLO} amplitude increase in HC, in patients with MCS, and in one subject affected by UWS). In addition, such after-effects were strongly inter-correlated (VMI and PMd-M1, VMI and SMA-M1, PPC-M1 and ERP_{LT} amplitude) and paralleled by a transient visuomotor CRS-R score improvement in some patients (MCS n. 4 and 5; UWS n. 3). In detail, some patients moved from the "perceptual awareness" (i.e. the potential to perceive the external world and to interact with it, expressed at least by the visual startle at the CRS-R) to a transient "visual consciousness" (at least visual fixation at the CRS-R). Nevertheless, since both some patients with UWS and MCS showed a stable visual fixation at the CRS-R (a score of 2 at visuomotor domain), the visual fixation could not be *per se* considered as a marker of MCS, as also suggested by functional neuroimaging studies (e.g. Bruno et al., 2010). On the other hand, we may argue that even UWS could have a residual potential to interact with external word through VMI processes, as also suggested by other studies concerning different sensory modalities, including pain processing (de Tommaso et al., 2015). Notably, a "general visual consciousness" (the highest grade of the VMI process that also enroll other extra-visual circuitries) needs more functional and structured visuomotor networks (O'Regan and Noë, 2011), which were not assessed by our protocol.

Hence, the after-effects of our real_protocol may suggest the possibility of a visuomotor output facilitation leading to a higher conscious level, even in some patients affected by UWS. This finding may be due to a potentiation of the residual premotor-motor, visuomotor, and visuo-cognitive functions,

thus suggesting a diagnosis of fLIS. Indeed, the preserved connectivity and the covert awareness may subtend a covert partial consciousness even in some patients with UWS (i.e. fLIS), as previously hypothesized (Laureys et al., 2010; Giacino and Zasler, 1995; Bekinschtein and Manes, 2008; Monti et al., 2013; Perrin et al., 2006). Classic and total LIS are instead characterized by nearly-normal brain connectivity.

4.1. tDCS-tACS physiology

A recent VEP-rTMS protocol induced a LTP/LTD-like plasticity within visuomotor areas (Suppa et al., 2015), probably by means of a topographically specific spike-timing dependent plasticity (STDP) within M1. Moreover, several studies have shown that tDCS may also induce cortical polarity changes within M1in a LTP-likewise (involving voltage-gated sodium channels and glutamatergic NMDA receptors) (Rizzo et al., 2014; Liebetanz et al., 2002; Nitsche et al., 2005; Ridding et al., 2000; Ridding and Uy, 2003; Kaelin-Lang et al., 2002; Naro et al., 2015a). Thus, our paradigm could have induced a LTP-like potentiation by means of hetero-synaptic STDP mechanisms within M1 (involving visual -tACS- and motor -TMS-inputs). The induction of a STDP mechanism is further corroborated by the lack of substantial effects following either the tDCS_alone, or the tACS_alone, or the sham_protocol. The after-effects we observed seem therefore to depend on the combination of the tDCS and tACS protocols.

Concerning the neural pathways supporting our after-effects, several studies have proposed that primary visual cortices could firstly elaborate the visuo-spatial information to generate visual perception and rapid-onset response (in analogy to other sensory-motor integration processes) (Keliris et al., 2010; Leopold and Logothetis, 1996; Saron et al., 2001; Tokimura et al., 2000; Valeriani et al., 1999; Sowman et al., 2014). Then, visual information may flow through extra-striate, temporal, parieto-occipital, DLPFC, premotor, and subcortical regions (maybe cerebellum and basal ganglia), in order to generate spatial localization processing, oculo-motor control, visual attention, motor planning and execution, and visually-guided motor command (Kravitz et al., 2011).The activity within the frontal, temporal, and parieto-occipital areas may oversee the conscious contents of visual information, whereas premotor and motor areas may organize conscious motor planning (Dolce et al., 2011). Hence, we may hypothesize that our protocol enrolled both dorsal and ventral visual pathways (Goodale and Milner, 1992), mainly involving the PPC and SMA (that have an updating effect of on visual feedback-based, active motor planning, motor attention, and motor intentions) (Desmurget and Grafton, 2000; Jeannerod et al., 1994; Ikkai and Curtis, 2011). In addition, we cannot exclude a possible role of the contralateral homologous areas and transcallosal connections, since we limited our measurements to the left hemisphere (Koch et al., 2009; Suppa et al., 2015).

Notably, we have to acknowledge other issues concerning the physiological effects of our combined real_protocol: i) meta-plasticity phenomena could play an important role in supporting and regulating our after-effects. Indeed, the sequential application of two tDCS protocols may have involved either homeostatic or non-homeostatic meta-plasticity mechanisms (Bienenstock et al., 1982; Davis, 2006), suggesting the preservation of stabilizing plasticity phenomena even in individuals with UWS; ii) Suppa et al. (2015) applied a pattern-reversal visual stimulation protocol (paired to rTMS) in an attempt to activate the left temporal hemiretina and focally trigger brain networks. We instead stimulated the entire left retina, and a double tDCS protocol was applied. Hence, we triggered brain networks with lower topographic specificity than the VEP-rTMS protocol; iii) since RMT, VEP and MEP parameters were not different at baseline between MCS and UWS, and RMT and VEP did not vary after the conditioning protocols, we can exclude the possibility that baseline cortical excitability or VEP differences could have influenced the after-effects. Moreover, in reason of the blinded condition of participants concerning the different experimental sessions, we may exclude differences in the attentive level in the HC participants (Stefan et al., 2004).

4.2. tDCS-tACS after-effects in patients with DOC

The potentiation of the M1 excitability, the premotor-motor connectivity, the visuo-cognitive function, and the visuomotor output in our patients may suggest the induction of VMI processes through time-locked neural activities that encompass premotor and parieto-occipital networks (spreading from the posterior to the anterior cortical areas and *vice versa*) and other subcortical areas (maybe including thalamus and basal ganglia) (Monti et al., 2013; Suppa et al., 2015; Thiebaut de Schotten et al., 2014; Bruno et al., 2010; Petrides and

Pandya, 2006; Di et al., 2014; Dum and Strick, 2005; O'Shea et al., 2007; Buch et al., 2010). Interestingly, it has been shown that visual pursuits depend on the activity of the mesiofrontal-precuneal cortex, which represents an important hub within the neural correlates of consciousness and is clearly impaired in patients suffering from severe chronic DOC (Bruno et al., 2010). The re-appearance or the enhancement of such visuo-motor responses could therefore express a functional upgrading -although transient - of the residual cortico-cortical and the brainstem-thalamo-cortical networks supporting VMI processes (Dolce et al., 2011; Bruno et al., 2010; Di et al., 2014; Riganello and Sannita, 2009).

It has been hypothesized that plasticity and connectivity recovery in individuals suffering from DOC might depend on the modulation of post-ischemic LTP (Crepelet al., 1993; Di Filippo et al., 2008), the production of specific neurotrophins (e.g. Brain-Derived Neurotrophic Factor; Kokaiaet al., 1998), and the regulation of excitatory/inhibitory dynamics within cortical and thalamo-cortical circuits (Dinget al., 2011). Thereby, it is conceivable that one or more of these mechanisms may have been triggered by the real_protocol, and could have favored the recruitment of silent or stunned cortico-cortical and cortico-subcortical connections, maybe involving the Schiff's mesocircuit model (Schiff, 2010).

Moreover, the bottom-up (stimulus salience) and top-down attention mechanisms (spatial and feature-driven attention, task goals) following the interaction between the ventral and the dorsal visual streams could have an important role in the clinical after-effects (Corbetta and Shulman, 2002; Currasco, 2011; Block, 2011; Pinto et al., 2013). Nevertheless, such issue needs to be further investigated by means of proper visuomotor tasks.

4.3. Study limitations, conclusions and future perspectives

In our opinion, our study proposes a promising approach in an attempt to identify residual patterns of VMI and large-scale fronto-parietal connectivity in patients affected by severe DOC, including UWS.

Noteworthy, the small sample size and the consequent mixed etiology represent a main limiting factor in our study. Nonetheless, it is difficult to study a large sample of patients with DOC, since the negative outcome of such patients is still unfortunately high.

However, our data further support the importance of patient's cooperation independent diagnostic approaches, aimed at assessing the consciousness level and differentiating real UWS from the patients that are clinically unable to express awareness signs (Formisano et al., 2013; Bruno et al., 2011; Schnakers et al., 2014; Naro et al., 2014). In addition, the possibility to identify such partially preserved cortico-cortical and cortico-subcortical networks in DOC may be useful in the selection of candidate patients for the deep brain stimulation (Schiff et al., 2007) or therapeutic and rehabilitative trials by means of non-invasive neurostimulation approaches.

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