Transcranial Alternating Current Stimulation Has Frequency-Dependent Effects on Motor Learning In Healthy Humans

Matteo Bologna,[a,b*] Andrea Guerra,[b*] Giulia Paparella,[a] Donato Colella,[a] Alessandro Borrelli,[a] Vincenzo Di Lazzaro,[c] Peter Brown,[d,e] and Alfredo Berardelli.[a,b]

[a] *Department of Human Neurosciences, Sapienza University of Rome, Viale dell'Università 30, 00185 Rome, Italy*
[b] *IRCCS Neuromed, Via Atinense 18, 86077 Pozzilli (IS), Italy*
[c] *Unit of Neurology, Neurophysiology, Neurobiology, Department of Medicine, University Campus Bio-Medico, Via Alvaro del Portillo 21, 00128 Rome, Italy.*
[d] *Nuffield Department of Clinical Neurosciences, University of Oxford, John Radcliffe Hospital, OX3 9DU Oxford, UK.*
[e] *Medical Research Council Brain Network Dynamics Unit, Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, UK.*

* Equally contributing authors

Corresponding Author:

Prof. Alfredo Berardelli

Department of Human Neurosciences,

Sapienza University of Rome

Viale dell'Università 30, 00185 Rome, Italy

E-mail : alfredo.berardelli@uniroma1.it

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ABSTRACT

It is well established that the primary motor cortex (M1) plays a significant role in motor learning in healthy humans. It is unclear, however, whether mechanisms of motor learning include M1 oscillatory activity. In this study, we aimed to test whether M1 oscillations, entrained by Transcranial Alternating Current Stimulation (tACS) at motor resonant frequencies, have any effect on motor acquisition and retention during a rapid learning task, as assessed by kinematic analysis. We also tested whether the stimulation influenced the corticospinal excitability changes after motor learning. Sixteen healthy subjects were enrolled in the study. Participants performed the motor learning task in three experimental conditions: sham-tACS (baseline), β-tACS and γ-tACS. Corticospinal excitability was assessed with single-pulse TMS before the motor learning task and 5, 15, and 30 min thereafter. Motor retention was tested 30 min after the motor learning task. During training, acceleration of the practiced movement improved in the baseline condition and the enhanced performance was retained when tested 30 min later. The β-tACS delivered during training inhibited the acquisition of the motor learning task. Conversely, the γ-tACS slightly improved the acceleration of the practiced movement during training but it reduced motor retention. At the end of training, corticospinal excitability had similarly increased in the three sessions. The results are compatible with the hypothesis that entrainment of the two major motor resonant rhythms through tACS over M1 has different effects on motor learning in healthy humans. The effects, however, were unrelated to corticospinal excitability changes.

INTRODUCTION

It is well established that the primary motor cortex (M1) plays a crucial role in motor learning, as evidenced in several animal studies (Nudo et al., 1996; Kleim et al., 1998; Plautz et al., 2000; Rioult-Pedotti et al., 2000; Tang et al., 2018). In humans, early studies using transcranial magnetic stimulation (TMS) showed that learning a simple motor task is associated with a functional reorganization of the M1, as assessed by corticospinal excitability changes (Pascual-Leone et al., 1994; Classen et al., 1998; Muellbacher et al., 2001, 2002; Bologna et al., 2015). Also, it has been reported that modulation of M1 excitability by repetitive TMS modifies motor learning in healthy subjects (Agostino et al., 2007, 2008; Hotermans et al., 2008; Sczesny-Kaiser et al., 2009; Iezzi et al., 2010; Censor and Cohen, 2011; Hamada et al., 2014; Platz et al., 2018). More recent investigations also suggested a role of M1 oscillatory activity in motor learning. For example, by recording cortical oscillations through various neurophysiological techniques, it has been observed that local changes in β and γ power, as well as modifications in coherence between sensorimotor areas, are both involved in various forms of learning (Manganotti et al., 1998; Miltner et al., 1999; Serrien and Brown, 2003; Perfetti et al., 2011; Nakano et al., 2013; Pollok et al., 2014). These data suggest that either the reduction of β oscillations or the increase of γ activity during training are associated with an improved behavioral outcome.

An innovative experimental approach to provide additional information in humans on the relationship between M1 rhythms and motor learning is to take advantage of the entrainment of brain oscillations produced by transcranial alternating current stimulation (tACS) (Antal and Paulus, 2013). Notably, when applied on brain regions at their resonant frequencies tACS induces coherent changes in the firing rate and timing of populations of neurons (Fröhlich and McCormick, 2010; Reato et al., 2013; Helfrich et al., 2014). It is also known that the influence of tACS on neuronal circuitries of M1 has a frequency-specific effect on motor execution (Pogosyan et al., 2009; Joundi et al., 2012; Wach et al., 2013; Guerra et

al., 2016, 2018b; Moisa et al., 2016; Nowak et al., 2017). Whether modulation of β and γ rhythms, by tACS, also modifies motor learning and M1 excitability changes due to motor practice remains largely unknown. To date, tACS studies mainly investigated implicit forms of motor learning (Pollok et al., 2015; Krause et al., 2016; Sugata et al., 2018), which likely involve complex physiological mechanisms. Moreover, none of the above studies tested possible influences of tACS during a motor learning task on practice-related changes of M1 excitability.

In the present study, we investigated the effects of tACS on motor learning, consisting of repetitive rapid abductions of the index finger. We specifically studied this form of learning as it is directly related to M1 activation (Muellbacher et al., 2001, 2002; Yokoi et al., 2018). We tested the effects of tACS on both the acquisition and retention phases of motor learning which possibly imply alternative mechanisms (Censor and Cohen, 2011; Bologna et al., 2015). To see whether tACS delivered on M1 during motor training also influences excitability changes, we monitored the course of motor evoked potentials (MEPs) before and after tACS.

EXPERIMENTAL PROCEDURES

Participants

The study enrolled 16 healthy subjects (4 females, mean age ± 1 standard deviation - SD: 27.4 ± 3.9 years) with no history of neurological and psychiatric diseases or medication intake. The sample size was calculated according to the results of previous studies investigating the effects of tACS on motor learning (Pollok et al., 2015; Krause et al., 2016; Sugata et al., 2018). All subjects were right-handed, as assessed by the Edinburgh Handedness Inventory (Oldfield, 1971). None of the subjects had ever engaged in

professional training involving the hands or had contraindications for non-invasive brain stimulation, as described in international guidelines (Rossi et al., 2009; Rossini et al., 2015; Antal et al., 2017). All participants were naïve to the stimulation techniques and none of them reported adverse effects or fatigue during or after the experiments.

Motor learning task and kinematic recordings

The motor training task was adopted from previous studies (Agostino et al., 2007, 2008; Li Voti et al., 2014; Bologna et al., 2015). Participants were comfortably seated in a chair during the experimental procedures with the arms slightly abducted from the trunk (~45–50°), the elbow flexed (~90°) and the dominant forearm in the prone position. The dominant (right) forearm and wrist rested on a table. The dominant wrist was kept in a neutral position. The dominant thumb was slightly abducted, and fingers 2–5 adducted extended at the inter-phalangeal and flexed at the metacarpophalangeal joints (~70–80 °). Participants were first asked to keep their dominant index finger extended and in line with the forearm. Participants were then instructed to produce rapid abductions of their dominant index finger, so as to achieve the highest acceleration possible, in response (but not to react immediately) to a 'go' signal, given randomly at ~0.2 Hz, and to return to the neutral position. During the motor training, 150 movements were collected; 15 consecutive movements were considered as a trial and averaged (10 acquisition trials overall). Moreover, we collected one retention trial of 15 consecutive movements 30' after the end of motor training. A rest interval of ~30s was left between trials to avoid fatigue. Before starting the task, one practice trial was allowed for the participants to become familiar with the experimental setting.

Repetitive finger movements were recorded using an optoelectronic motion system (SMART motion system, BTS Engineering, Milan, Italy). This system comprises three infrared cameras (sampling rate, 120 Hz) that follow the 3D displacement of reflective markers taped to the participant's hand. We used five reflective markers (5mm in diameter) of negligible

weight. One marker was placed on the tip of the index finger. Three additional reflective markers were placed on the hand to define a reference plane that was used to mathematically exclude unwanted hand movements from the recording of index finger abductions (Li Voti et al., 2014; Bologna et al., 2015).

To quantify repetitive finger movement kinematics, we used a dedicated software (SMART Analyzer, BTS Engineering, Milan, Italy) to automatically reconstruct the 3D spatial displacement of the reflective markers off-line and the acceleration of index finger abductions. The first acceleration peak of each index finger abduction was expressed in acceleration of gravity (g) (Bologna et al., 2015).

Brain stimulation and electromyographic recordings

tACS was delivered through conductive rubber electrodes enclosed in saline-soaked sponges using a BrainSTIM (EMS, Italy) and secured in place using rubber strips around the head. The stimulating electrodes (5 × 5 cm) were placed over M1 and Pz, as detailed elsewhere (Feurra et al., 2011, 2013; Guerra et al., 2016, 2018a, 2018b). The setup was optimized in order to ensure that the impedance for stimulation was < 10 kΩ. tACS was delivered at two different frequencies: 20 Hz (β) and 70 Hz (γ), as in previous tACS studies (Pogosyan et al., 2009; Joundi et al., 2012; Moisa et al., 2016; Guerra et al., 2018a). We have chosen these specific frequencies as representative of the two major natural rhythms of M1 (as discussed in Pogosyan et al., 2009; Joundi et al., 2012; Moisa et al., 2016; Guerra et al., 2018a). A sham tACS stimulation was used as a control. During sham tACS the device was activated for only 7 seconds, consisting of 3 seconds of ramp up and down periods and 1 second of stimulation at 20 Hz (Guerra et al., 2018a, 2018b). Sine wave stimulation was delivered with no direct current offset and a peak-to-peak amplitude of 1 mA. In case this intensity induced significant skin discomfort or obvious visual sensations, the stimulation amplitude was gradually lowered until no side effects were present. As a result, participants

did not perceive any sensation during β, γ or sham tACS and they were, therefore, unable to distinguish among the different stimulation conditions. The resulting stimulation intensities in the three sessions were: 0.71 ± 0.06 mA for β tACS and 1.0 ± 0.0 mA for γ and sham tACS.

Transcranial Magnetic Stimulation (TMS) was performed by using MAGSTIM 200 (Magstim Company Limited, Whitland, South West Wales) connected to a standard figure-of-eight coil delivering monophasic pulses. The TMS coil was held with the handle angled at 45° to the midsagittal line, pointing posteriorly and laterally. The point from which TMS stimuli at the minimal intensity elicited MEPs of maximal amplitude (i.e. 'hotspot') in the right first dorsal interosseous (FDI) muscle was then targeted. The MEPs were recorded through a pair of surface electrodes placed on the FDI muscle of the dominant hand in a belly/tendon montage. Resting motor threshold (RMT), i.e. the stimulator's output able to elicit MEPs of ≥ 50 μV peak-to-peak amplitude in at least 5 out of 10 consecutive stimuli, was determined as well as the minimum intensity needed to reliably produce MEPs of about 1 mV in size (1mV-MEP).

Electromyographic (EMG) signals were amplified by a Digitimer D360 amplifier (Digitimer Ltd, Welwyn Garden City, UK), digitized at 5 kHz (CED 1401 laboratory interface; Cambridge Electronic Design, Cambridge, UK), and analyzed off-line with a dedicated software (Signal software version 5.08; Cambridge Electronic Design).

Experimental design

Participants underwent three main sessions consisting of motor training performed during different modalities of tACS delivered on M1 (sham, β, and γ). The three sessions were performed at least one week apart, and the session order was randomized and counterbalanced between subjects. Importantly, tACS was activated about ten seconds

before starting the motor training, it was left ON for the whole duration of the task (i.e. 15 minutes) and then switched immediately OFF.

Stimulations at an intensity evoking 1 mV amplitude MEP (1 mV-MEP) were performed in all the three main sessions at four measurement time points: baseline (before motor training) and 5', 15' and 30' thereafter (Figure 1a). Twenty single pulse MEPs were recorded at each time point.

Aiming to verify whether the possible effects of tACS on motor learning were due to concurrent changes in corticospinal excitability we performed a control experimental session on 13 subjects of the original sample of 16 participants (4 females; mean age 27.5 ± 5.1 years). In particular, we tested the effects of β and γ tACS (delivered alone, without motor training) on M1 excitability by recording 20 1mV-MEP by single-pulse TMS at baseline (before tACS), at two time-points during the stimulation (during-5' and during-10') and at three time-points after the end of the stimulation (post-5', post-15' and post-30') (Figure 1b). The stimulation lasted the same time as the duration of the motor training task performed in the main experiment (i.e. about 15'). In the control experiment, the targeting of the FDI 'hotspot' by TMS was firstly performed on the scalp and then repeated over the sponge, where the site was marked with a felt-tip pen to facilitate a correct coil repositioning during the MEP collection. The two sessions of the control experiment were randomized and performed with a time interval of at least one week. In all experiments the participants and the researchers who collected and analyzed kinematic measures and MEPs were all blinded to the stimulation conditions. There was only one researcher unblinded to the stimulation conditions, the one who applied tACS and set up the stimulation frequencies.

Data analysis and statistics

To quantify the acquisition of the task during motor training we considered the changes in the acceleration peak of index finger abductions in each trial of the motor training. As an index of acquisition of the practiced movement, i.e. ONLINE effect, we determined the slope of the regression line interpolating the curve of the index finger acceleration changes during the ten trials of motor training. To quantify how participants retained the practiced movement, i.e. OFFLINE effect, we measured the ratio between the mean acceleration peak of the motor retention trial and the mean acceleration peak of the 10^{th} trial of the motor learning task (Cantarero et al., 2013a, 2013b; Bologna et al., 2015). To quantify the corticospinal excitability before and after motor training we measured peak-to-peak amplitude of MEPs.

To analyse the course of the acceleration peak during the motor learning task, absolute values (g) were entered into a repeated-measures analysis of variance (rmANOVA) using SESSION (three levels: sham, β, and γ) and TRIAL NUMBER (ten levels: 1^{st} to 10^{th} trials) as a within-subject factor. We also assessed the data concerning the ONLINE effect and the OFFLINE effect in two additional rmANOVAs using SESSION as within-subject factor. To analyse the MEP amplitude changes before and after motor training in the three conditions we performed a rmANOVA using SESSION (three levels as for the acceleration peak) and TIME POINT (four levels: before the motor learning task and 5', 15' and 30' after MT) as within-subject factor. To analyse the MEP amplitude changes during β- and γ-tACS delivered alone we performed another rmANOVA using SESSION (two levels: β and γ) and TIME POINT (six levels: before tACS, during-5', during-10', post-5', post-15' and post-30') as within-subject factors. Bonferroni's test was used for post-hoc analyses in ANOVA.

Pearson's product-moment correlation coefficient was adopted to test possible correlations between the ONLINE effect and the practice-related excitability changes of M1 in the three experimental sessions. Accordingly, the latter parameter was measured as the average

increase in MEP size at 5', 15' and 30' post-motor training (values normalized for their baseline, i.e. before motor training). Pearson's correlation also was used to evaluate whether possible tACS-induced changes of motor training (ONLINE effects) were associated with corticospinal excitability changes during tACS. For this purpose, the MEP amplitude during β and γ tACS (average of measures recorded at 5' and 10') were normalized by their corresponding baseline (pre-tACS values).

Unless otherwise stated, all results are indicated as mean values ± 1 standard error of the mean (SEM). Data normality was analysed by means of the Kolmogorov-Smirnov normality test. In all tests, the level of significance was set at $P<0.05$. Data were analyzed using STATISTICA® (StatSoft, Inc).

RESULTS

Motor training and kinematic recordings

Figure 2a depicts the course of the acceleration peak throughout the 10 trials of the motor learning task and in the retention trial in the three sessions of the main experiment (γ tACS: $R=0.86$, $P=0.001$; sham tACS: $R=0.79$, $P=0.005$; β tACS: $R=0.47$, $P=0.17$); Acceleration values in the first trial of the motor training were similar across the three conditions (all $Ps>0.05$, Figure 2a). The rmANOVA showed a significant effect of TRIAL NUMBER ($F_{9,135}=6.30$, $P<0.01$), indicating that participants increased their acceleration during training. Although there was no significant effect of SESSION ($F_{2,30}=0.68$, $P=0.51$), we found a significant TRIAL NUMBER x SESSION interaction ($F_{18,270}=2.94$, $P<0.001$), suggesting that participants improved their motor performance at different rates in the three training sessions of the main experiment. This result was confirmed in three separate follow-up rmANOVA showing a significant effect for the TRIAL NUMBER for the γ session ($F_{9,135}=7.22$, $P<0.001$)

and for the sham session ($F_{9,135}$=3.52, P=0.006) but not for the β session ($F_{9,135}$=1.85, P=0.07). Post hoc analysis showed that during the sham session subjects improved their performance only in the last two trials of the task (1st vs. 9th: P<0.001; 1st vs. 10th: P=0.004). In contrast, the acceleration peak significantly increased early in the course of the task, i.e. starting from the second trial, in the γ session (1st vs. 3rd - 10th: all Ps<0.05).

The analysis we performed on the ONLINE-effect showed that the main factor SESSION was significant ($F_{2,45}$=6.32, P=0.003). Post-hoc analysis indicated that the acquisition of the practiced movement was lower in the β session compared to sham (P=0.04) and γ (P=0.003) sessions; whereas no difference was present between sham and γ sessions (P=0.92). Similarly, the analysis we performed on the OFFLINE-effect also showed a significant main factor SESSION ($F_{2,45}$=6.52, P=0.003). Post-hoc analysis demonstrated that retention of the practiced movement was reduced in γ (γ vs. sham: P=0.04; γ vs. β: P=0.01), while sham and β were not different (P=0.99) (Figure 2b).

As the participants performed three randomized sessions, we excluded the possible occurrence of carry-over effects on consecutive measurements using a repeated measures ANOVA using SESSION ORDER (three levels: 1st, 2nd, and 3rd) and TRIAL NUMBER (1^{st} to 10^{th} trials) as within-subject factors. The analysis revealed no significant effects of the main factor of analysis SESSION ORDER ($F_{2,30}$=1.97, P=0.16) and no significant SESSION ORDER x TRIAL NUMBER interaction ($F_{18,27}$=0.57, P=0.92).

In summary, the results indicate that participants' performance during motor training was frequency-dependently modulated by tACS. Namely, β-tACS had a detrimental effect on motor learning acquisition. In contrast, γ-tACS improved motor performance early during motor training but it had a detrimental effect on motor retention, with respect to the baseline.

Measures of M1 excitability

Baseline measurements of motor thresholds are summarized in Table1. RMT ($F_{2,30}$=0.35, P=0.71) and 1mV-MEP values ($F_{2,30}$=0.15, P=0.86) did not differ between sessions.

As shown in Figure 3a, the overall excitability of the dominant FDI area increased after motor training in the three main sessions. This was confirmed by rmANOVA, which showed a significant effect of TIME POINT ($F_{3,45}$=24.35, P<0.001). Post-hoc analysis revealed that M1 excitability was enhanced 5' (baseline vs. post 5': P<0.001), 15' (baseline vs. post 15': P<0.001) and 30' after motor training (baseline vs. post 30': P=0.02). The analysis, however, revealed no significant effect of SESSION ($F_{2,30}$=0.016, P=0.98) and no interaction SESSION x TIME POINT ($F_{6,90}$=0.42, P=0.86).

The rmANOVA on MEPs recorded in the two sessions of the control experiment showed no significant effect of the main factors SESSION ($F_{1,12}$=1.38, P=0.26) and TIME POINT ($F_{5,60}$=1.15, P=0.34) and no SESSION x TIME POINT interaction ($F_{5,60}$=0.52, P=0.76). (Figure 3b).

To sum up, training fast index finger abductions increased the excitability of the FDI area. However, the effects on the M1 excitability did not differ when motor training was accompanied by tACS, delivered at different frequencies. Finally, the results indicate that β and γ tACS alone did not modify the M1 excitability during stimulation and did not induce after-effects.

Intensity-dependent effects of β-tACS

Since the stimulation intensity of β tACS (0.71 ± 0.06 mA) was on average lower than that used for γ tACS (1.0 ± 0.0 mA) we investigated whether the effects of β tACS on movement kinematics or practice-induced changes of corticospinal excitability correlated with the stimulation intensity used. We found no significant correlation between the stimulation intensity during β tACS and the ONLINE effect (r=-0.11, P=0.67) or practice-induced

changes of corticospinal excitability (average of 5', 15' and 30' post-training) (r=0.13, P=0.63).

Correlation analysis

The analysis demonstrated that no relationship was present in any of the three experimental sessions between the ONLINE effect and the level of practice-induced changes of corticospinal excitability (sham: r=-0.04, P=0.88; β: r=0.14, P=0.6; γ: r=-0.32, P=0.22). Thus, our data suggest that practice-induced changes of corticospinal excitability do not depend on changes in motor performance due to learning.

Also, we tested whether the modulation of the index finger acceleration during the motor learning task correlated, at the individual level, with MEP amplitude recorded during β and γ tACS. We found no relationship between changes in the ONLINE effect and M1 excitability either during β tACS (r=-0.16, P=0.60) and γ tACS (r= -0.10, P=0.97). We may thus conclude that the frequency-dependent effects of tACS on motor behaviour were unrelated to individual M1 excitability changes.

DISCUSSION

In the present study, we found that tACS influences the acquisition phase of motor learning in a frequency-dependent manner. β tACS reduced motor learning, as evidenced by failure to improve motor performance during training. Conversely γ tACS produced a slightly improved motor performance with respect to baseline condition but with a detrimental effect on motor retention. Also, neither β nor γ tACS influenced practice-related excitability changes in M1. Finally, correlation analysis further confirmed that the frequency-dependent effects of tACS on motor behaviour were unrelated to M1 excitability changes.

The effects we observed are not simply explained by different levels of baseline performance in the training sessions since participants had comparable index finger acceleration in the first trial of the motor task. We can also exclude that our results might be influenced by differences in baseline corticospinal excitability since this was similar in the three sessions of the main experiment. Moreover, it is unlikely that the observed results are influenced by any carryover effects of the interventions we used as the three main experimental sessions were randomized and performed at least one week apart and these were also ruled out in ad-hoc analysis. Two previous studies have suggested that tACS can modulate attentional networks (Hopfinger et al., 2017; van Schouwenburg et al., 2018). However, these studies used simulation at different brain sites and at different frequencies to the current study, which focussed on stimulation over M1. Finally, it seems unlikely that subjects could distinguish between the different stimulation regimes, as they were blinded to condition, stimulation was set to be below threshold for paraesthesiae and flashes, sessions were separated by at least a week and there was no difference in task performance in the first trial of each session. Accordingly, we propose that entrainment of β and γ rhythms in M1 by tACS, a process following from the 'resonance principle' (Schutter and Hortensius, 2011), affects the physiological processes developing or sustaining improvements in motor performance during training.

We found that the β tACS reduced movement acceleration throughout the motor task by flattening the slope indicative of improving motor performance over training. The difference between beta and gamma/sham sessions thus became more pronounced in the last part of the training. Because previous studies demonstrated that β tACS may exert antikinetic effects on voluntary movements (Pogosyan et al., 2009; Joundi et al., 2012; Guerra et al., 2018a), one possible explanation of our findings is that stimulation interfered with movement execution processes with a temporal delay (Wach et al., 2013). Nevertheless, β tACS did

not modify the movement acceleration in the first trial of the training and the overall amount of acceleration in the β tACS session did not significantly change across trials. Thus, we favour the alternative hypothesis that tonically increasing the oscillatory activity of M1 in the β band through tACS possibly interferes with endogenous β rhythms and cortical reorganization associated with motor learning. Supporting this hypothesis, a MEG study showed that the amount of β suppression specifically represents a neurophysiological marker of early cortical reorganization associated with motor learning (Pollok et al., 2014). It has been also reported by using EEG that changes in β rhythms in sensorimotor areas occurring during observation, preparation, and repeated movement execution are associated with motor skill improvement (Manganotti et al., 1998; Nakano et al., 2013). Specifically, a reduction of β oscillatory activity during action execution is most prominent in subjects with the highest learning performance (Manganotti et al., 1998).

In contrast to β tACS, γ tACS produced a slight improvement of acceleration during training, but no significant increase in the slope of motor performance, and moreover had a detrimental effect on motor retention, with respect to baseline conditions. These results overall indicate that the improved motor performance during γ tACS does not necessarily imply that the stimulation potentiates the physiological mechanisms of motor learning. Rather, it might be that γ tACS interferes with the mechanisms of motor retention. To date, there is only limited evidence suggesting a possible relationship between γ oscillations of M1 and motor learning. Previous studies have shown that increased activity in the γ band within M1, as well as γ band coherence between motor areas, are both possible mechanisms involved in associative learning procedures in humans (Miltner et al., 1999; Serrien and Brown, 2003). In our study, however, we tested a simple form of motor learning which possibly involves different physiological mechanisms.

Unlike motor behaviour during training, tACS delivered at β or γ frequencies to M1 did not affect practice-related changes in corticospinal excitability. These results indicate that modulation of behavioural outcome occurred independently from practice-related corticospinal excitability changes. In this regard, the results of previous reports (Bologna et al., 2015; Hussain et al., 2016), support the hypothesis that changes in behavioural motor outcome are not necessarily paralleled by practice-dependent changes in corticospinal excitability. It is also worth considering that the lack of effects of tACS on practice-related corticospinal excitability changes differs from those we previously reported using intermittent theta burst stimulation (iTBS) (Guerra et al., 2018b). In our previous study we found that iTBS-induced plasticity of M1 can be significantly boosted by the concomitant application of γ tACS. The results of the two studies can be explained under the assumption that the mechanisms underlying endogenous (e.g. due to motor practice) and exogenous (e.g. TMS-induced) brain plasticity are not necessarily the same (Guerra et al., 2017a, 2017b). Thus, the data point toward the possibility that different forms of M1 plasticity are variably susceptible to changes of γ oscillations.

The present study has several limitations that should be mentioned. Gender ratio was slightly unbalanced in favour of male preponderance; due to the lack of specific studies on the effects of gender on tACS we don't know whether this aspect could have influenced our results. Importantly, considering the limited topographic specificity of tACS, we cannot entirely exclude that the effects we observed are due to modulation of a wider network including connections with interconnected brain areas like the somatosensory cortices, basal ganglia or cerebellum. Future modelling of current distributions might be helpful in determining the extent of possible tACS effects. This might also help inform whether the lower intensity of stimulation in the beta session was sufficient to influence M1, although the correlational analysis performed in the present study failed to demonstrate any intensity

dependency of the ONLINE effect. Since this study specifically assessed corticospinal excitability using single-pulse TMS, we also cannot exclude that other physiological mechanisms, like intracortical excitability, known to be influenced by β and γ tACS (Guerra et al., 2016; Nowak et al., 2017; Guerra et al., 2018b), underpinned the behavioural changes. In addition, we did not record EEG activity prior the task and therefore we did not stimulate at individual β and γ peak frequencies, an approach that could improve the reliability of the results. Equally important, we did not record EEG activity during the task to directly demonstrate modulation of β and γ oscillations by tACS at corresponding frequencies. Unfortunately, such recordings are confounded by artefact related to stimulation which is orders of magnitude greater than the endogenous rhythmic activity. Finally, we focused on the two major motor resonant rhythms, and the assessment of the possible role of activity in other frequency domains, such as the α band (Nakano et al., 2013; Pollok et al., 2014, 2015), was beyond the scope of the study.

In conclusion, this study provides novel information suggesting that tACS, delivered at motor-resonant frequencies, influences motor learning in healthy humans in a frequency dependent manner. Modulation of behavioural motor outcome, however, is not driven by simple changes of corticospinal excitability during stimulation and occurs independently from practice-related excitability changes. Our study provides novel information useful for a better understanding of the physiological relationship between cortical oscillations and plasticity in healthy subjects. Further studies are needed to clarify the effects of tACS either on more complex forms of motor learning in physiological conditions or on altered motor learning in neurological disorders.

CONFLICT OF INTEREST AND FUNDING SOURCES

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FIGURE CAPTIONS

FIGURE 1. Experimental Design.

The upper panel (a) shows the design of the three randomized sessions of the main experiment. Participants performed the motor learning task (MLT) during different modalities of tACS (gamma, sham, and beta) delivered on M1. The motor evoked potentials (MEPs) by 20 single pulse TMS were recorded before the motor learning task (pre MLT) and 5, 15 and 30 min thereafter (post MLT) at an intensity evoking 1 mV amplitude MEP (1 mV-MEP). Immediately after the 30 min MEP recording one retention trial was also collected. As shown in the schematic representation of the experimental setting, tACS electrodes were positioned on the M1 'hotspot' of the dominant hemisphere (stimulating electrode) and on Pz (reference electrode), and their edges were separated by a minimum of about 2-3 cm. Note that the wire connection location is only illustrative.

The lower panel (b) shows the design of the two randomized sessions of the control experiment. In this experiment the effect of gamma and beta tACS delivered alone (without MLT) on corticospinal excitability was tested. MEPs by single pulse TMS were recorded at the intensity of 1 mV-MEP at baseline (pre tACS), at two time-points during the stimulation (during tACS 5 min and 10 min) and at three time-points after the end of the stimulation (post tACS 5 min, 15 min, and 30 min).

FIGURE 2. Effects of tACS on motor learning.

The upper panel (a) shows the group mean acceleration peak + 1 standard error of the mean during the 10 trials of the acquisition phase and in the retention trial of the motor learning task (MLT) in the three stimulation conditions (gamma, sham, and beta). Note that motor performance improved during sham and gamma, but not during beta tACS.

The lower panel (b) shows the group mean ONLINE (left) and OFFLINE (right) effects in the three main sessions (gamma, sham, beta). The ONLINE effect (slope of acceleration changes during the motor training) was markedly lower in the beta session with respect to sham and gamma. The OFFLINE effect (ratio between the acceleration of the retention trial and the acceleration of the 10^{th} trial of the learning task) was lower in the gamma session with respect to sham and beta. Note that no difference between sham and beta was present, but this could be explained by the flattened course of the acceleration in the beta session. Note also that the ONLINE and OFFLINE effects are presented for contrasting between sessions. ONLINE and OFFLINE effects are not intended to be compared to one another as they are derived in different ways.

FIGURE 3. Effects of tACS on corticospinal excitability.

The upper panel (a) shows the group mean MEP amplitude (y axis) + 1 standard error of the mean in the four measurements time points (x axis) in the three sessions of the main experiment. Note that MEP amplitude was comparable at baseline (pre MLT) and increased to a similar extent after the motor training (5, 15, 30 min post MLT) in the three different sessions.

The lower panel (b) illustrates the lack of MEP amplitude changes before (pre tACS), during (5 and 10 min) and after (post 5, 15 and 30 min) gamma or beta tACS in the control experiment.