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PhD Dissertation

THE PLACEBO EFFECT IN THE MOTOR DOMAIN: A NEURAL AND BEHAVIORAL APPROACH

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La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante.

(True science teaches, above all, to doubt and be ignorant.)

Miguel de Unamuno

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PREFACE

The placebo effect is a beneficial outcome that follows the administration of a treatment and that is not to be ascribed to active ingredients but to the words, contexts and beliefs that surround the treatment and that can induce psychological and neuronal changes in the recipient's brain (Benedetti et al., 2011). This psychobiological phenomenon represents a good model to study the mind-brain-body interaction.

During the past decades, the placebo effect has attracted the interest of researchers from different backgrounds, such as psychology, neurobiology, cognitive neuroscience among others. Many important studies in the healing context, adopting clinical trials or experimental models of pain, have allowed to achieve a deep understanding of the neurobiological correlates and cognitive mechanisms involved in this phenomenon. In more recent years, however, it has become always clearer that the placebo effect is a pervasive phenomenon that extends beyond the healing context (Pollo et al., 2011). With regard to this, different lines of evidence have shown that the placebo effect can be found in many contexts, such as the cognitive, the sensory, the emotional and the motor domains (Beedie & Foad, 2009; Beissner et al., 2015; Schienle et al., 2013; Schwarz & Büchel, 2015).

During my Ph.D. I have been particularly interested in enlarging our knowledge on the placebo effect in the motor domain. This interest derives from scientific curiosity, as well as from the potential future translational impact of the motor placebo effect in sports and pathology. For instance, it could be possible to think at the placebo effect as a strategy to implement the outcome obtained with the traditional sport trainings and also as a complementary strategy for motor recovery, for instance in patients in whom the pharmacological treatment is less effective. However, before achieving this translational impact, some issues need to be clarified: First, the neural correlates of the placebo effect in the motor domain are still largely unknown; Second, knowledge is still limited on the type of motor functions that could be influenced by the placebo effect.

With regard to the first issue, up-to-now very few studies have investigated the neural correlates of the motor placebo effect in healthy participants. These studies highlighted the role of the primary motor cortex (M1) and the supplementary motor

area (SMA) (Fiorio et al., 2014; Piedimonte et al., 2015). Different approaches in patients affected by movement disorders, like Parkinson's disease, hinted at the involvement of subcortical structures, like the subtalamic nucleus (STN), the substantia nigra pars reticulata (SNr), the ventral anterior (VA) and the anterior ventrolateral (VLa) nuclei of the thalamus (Benedetti et al., 2004; Benedetti et al., 2009). All these brain areas are not isolated, but are strongly connected with other brain regions, such as the dorsolateral prefrontal cortex (dlPFC) (Hasan et al., 2013; Mayberg et al., 2002; Miller & Cohen, 2001), the cingulate cortex (Asemi et al., 2015; Mayberg et al., 2002; Petrovic et al., 2002), or the orbitofrontal cortex (Petrovic et al., 2002) among others. Of note, these areas have been consistently shown to be involved in placebo analgesia (Ashar et al., 2017; Wager & Atlas, 2015) and it is reasonable to hypothesize that they could also play a role in the motor placebo effect. In particular, the dlPFC plays a crucial role in placebo analgesia and it is also involved in higher-order cognitive functions, like expectation and anticipation, that are at the basis of the placebo effect. Moreover, the dlPFC also has connections with motor brain areas, thus suggesting a potential role of this brain region in the motor placebo effect. Part of my Ph.D. project was dedicated to tackle the role of the dlPFC in the motor placebo effect in healthy participants. In *Part II* of this thesis, I will describe a series of three experiments in which we applied non-invasive brain stimulation combined with a placebo procedure on force production. This investigation has allowed to enlarge the current knowledge on the neural correlates of the placebo effect in the motor domain, by demonstrating that the dlPFC could also play a role, especially when expectation is the main cognitive mechanism at the basis of the placebo effect.

With regard to the second issue, so far, the behavioral investigation of the placebo effect has addressed some dimensions of motor performance, such as force production, movement speed and resistance to fatigue (Beedie & Foad, 2009; Fiorio et al., 2018; Pollo et al., 2011). The potential effects of placebo on other crucial motor functions remains unknown. Motor performance is a complex definition that embraces different dimensions, such as precision control, balance, visuomotor coordination, motor sequence learning and motor adaptation, among others. Some of these motor functions are important not only in sports, but also in daily life

activities. Hence, extending the behavioral investigation of the placebo effect on other motor functions may help to achieve a better understanding of the placebo effect itself, as well as to enlarge its range of application to daily life activities. During my Ph.D., I have tried to explore whether a placebo procedure can improve two relevant motor functions present in our daily life, like balance control and motor sequence learning. Balance can be defined as the capacity *"to control the body's position in space for stability and orientation*" (O'Sullivan, 2007). Balance control is crucial to accurately perform most of the activities in daily life, such as getting up from bed, walking, waiting on a queue or simply having a shower. Conversely, disturbances in balance control, such as those present in Parkinson's disease or in the elderly population can lead to higher risk of fall, which limits the quality of life (Jacobs et al., 2005; Maki et al., 1994; Pfortmueller et al., 2014). In *Part III* of this thesis, I will describe a new protocol that we developed to improve balance control in healthy participants with a placebo procedure. We think that extending the potential beneficial effects of placebos on balance control may allow to provide in the future new strategies for the rehabilitation of gait disorders for which the pharmacological treatment is often not effective.

Motor skill learning in another important motor function that permits to convert isolated and specific movements into well-performed skills through practice (Dayan & Cohen, 2011; Wolpert et al., 2011). In particular, motor sequence learning is crucial in many tasks, such as cooking and cleaning, and to acquire skills, like writing or cycling that are present during the lifespan. In *Part III* of this thesis, I will describe a study that we conducted to investigate the placebo effect on the learning of motor sequences in healthy participants. Even in this type of study, we envisage a potential future impact for rehabilitative trainings after an injury (i.e. stroke) (Kitago & Krakauer, 2013).

To summarize, with this work we have tried to expand the current knowledge on the placebo effect in the motor domain from two different perspectives: from one hand, we have conducted a neural investigation on the involvement of a higher-order brain region (the dlPFC) in the motor placebo effect; from the other hand, we have enlarged the behavioral investigation of the placebo effect to two different movement functions: balance control and motor sequence learning.

ABSTRACT

The placebo effect is a fascinating psychobiological phenomenon that allows to investigate the mind-body interaction. It is typically induced by the application of an inert treatment along with verbal suggestion of beneficial outcomes. The placebo effect has been deeply investigated in the field of pain, although different lines of evidence suggest that it is also present in other domains, like the motor domain. Extending our knowledge of the placebo effect in the motor domain can have important future translational impacts in sports and pathology. The aim of my PhD project was to study the placebo effect in the motor domain at two different levels: the neural and the behavioral level.

Regarding the neural level, knowledge on the brain regions related to placebo effect in the motor domain is limited. We aimed at filling in this knowledge gap by investigating the role of the dlPFC, a brain region also involved in placebo analgesia. The dlPFC elaborates expectation, a cognitive function at the basis of the placebo effect and shares some connections with other brain regions involved in motor control. Hence, there are many clues to hypothesize a role of the dlPFC in the motor placebo effect. To tackle this issue, three different experiments were conducted in which the dlPFC was stimulated by means of transcranial direct current stimulation (tDCS) together with a placebo procedure on force production. We found that the left dlPFC is involved in the expectation-induced enhancement of force, specifically in those subjects who respond to the placebo effect (placebo-responders).

Regarding the behavioral level, it should be noticed that many behavioral studies have shown that the placebo effect can enhance different aspects of motor performance associated to sports, such as force, speed or endurance. It is still unknown, however, whether the placebo effect can also improve other motor functions, important for many daily life activities, like balance or motor sequence learning. Thus, another objective of my PhD was to investigate the potential influence of the placebo effect on two motor functions that are closer to daily life activities. To this aim, a first study was conducted to understand whether balance control, a motor function needed for many daily life activities and for preventing falls, could be enhanced in healthy participants by a placebo procedure consisting

of verbal suggestion. We found that different parameters of balance (in the three-dimensional space and in the medial-lateral direction) and the subjective perception of stability were improved by the placebo procedure.

A second behavioural study was run to investigate whether the application of a placebo treatment consisting of verbal suggestion could help in improving motor sequence learning. In this case, we also aimed to tackle a differential role of two types of placebo treatments: one motor and one cognitive. The motor placebo procedure consisted of transcutaneous electrical nerve stimulation (TENS) applied the hand muscles involved in the task together with verbal information on the beneficial effects on muscle activity. The cognitive placebo procedure consisted of sham transcranial direct current stimulation (tDCS) applied over the frontal region together with verbal information on the beneficial effects on attention. Our findings did not show a clear improvement of performance following the placebo procedures, but a significant effect on the subjective perception of fatigue. More precisely, while the placebo procedure directed to the motor function (TENS) could reduce the perception of physical fatigue, the placebo procedure focused on cognitive functions (sham tDCS) could decrease the perception of both mental and physical fatigue.

Altogether these investigations represent an attempt to deepen our understanding of the neural correlates of the motor placebo effect and to enlarge the potential behavioural influence of placebos on different motor functions.

PART I: BACKGROUND

Definition of the placebo effect

Placebo is a latin word that means "I shall please" and it was first documented in the Vespers for the Dead (Psalm 116, 9th verse) as *Placebo Domino in regione vivorum* (Hart, 1999; Jacobs, 2000). By that time, the term placebo started to be used with the notion of "pleasing". For example, some mourners were hired to "sing placebos" to adulate the dead at the burials (Kerr et al., 2008).

Some years had to elapse to find the word placebo associated to the medical lexicon. During the late 18th century, a British physician used the word placebo for describing a method to give comfort and please to patients with illnesses without cure (Kerr et al., 2008), thus associating the word to console or relieve more than to cure. Several years later, in 1811, a new explanation of the word placebo was registered and defined in the Hooper's Medical Dictionary. Placebo was defined as *"any medicine adapted more to please than benefit the patient"* (Kerr et al., 2008; Finniss, 2018).

During the 20th century, the term placebo started to be slightly transformed and associated to therapeutic rituals and deception (Carlill, 1918). But it was during the mid-20th century when the word placebo started to be associated with the placebo effect. In 1955, Henry K. Beecher discovered the effect of placebo by using a placebo-controlled double-blinded design, using the placebo as a tool to dissociate the real pharmacological effect from the suggestion that arises when a pharmacological treatment is applied. In his article, the placebo resulted effective in the 35.2% of the cases and the effect was associated to the subjective component that emerges from the applied treatment. Several subsequent researches have attempted to obtain a better definition of the placebo effect (Benedetti, 2002; Shapiro, 1997; Vase, 2002).

A related aspect to consider is the difference between the terms placebo effect and placebo response that are usually considered interchangeable in the literature, although a difference exists between these two terms. According to Hoffman et al. (2005), the term *placebo response* refers to the individual change that is caused by

a placebo manipulation or treatment simulation, while the term *placebo effect* refers to the average enhancement that occurs in a group of subjects after receiving a placebo manipulation or treatment simulation.

Today, the most accepted definition of the placebo effect refers to a psychobiological phenomenon that could be defined as a physical or psychological benefit following the administration of an inert substance (or sham treatment) together with a positive context inducing positive expectations about its effect (Benedetti et al., 2016).

Cognitive mechanisms of the placebo effect

Placebo responses are the result of a complex interaction of different biopsychosocial factors, such as cognitive functions like expectation and learning, personality traits and genetic factors (Colagiuri et al., 2015; Colloca et al., 2014; Colloca & Miller, 2011; Corsi & Colloca, 2017; Peciña et al., 2013). In particular, expectation and learning represent the main cognitive mechanisms implicated in the modulation and formation of the placebo effect.

The expectancy model is considered as the central mechanism for the development of the placebo effect and makes a special mention of the importance role of verbal suggestions (Colloca & Miller, 2011; Kirsch, 2018). According to the Theory of Expectancy (Kirsch, 1985), the previous belief of a person about what will happen in certain circumstances will determine what that person will experience in the end. Thus, a response (placebo) can occur as consequence of expecting a positive outcome. In other words, the expectation can be generated by positive verbal suggestions associated with a treatment (actually inert), thus inducing a positive expectation and creating a real effect. This induction of positive expectation about the effect of an ergogenic aid (actually inert) in reducing symptoms (i.e., pain) can be sufficient to modify pain perception, pain sensation and dopamine release (Benedetti et al., 2003) and can also modulate motor and cognitive performance (Beedie et al., 2009; Colagiuri et al., 2011).

Another crucial psychological mechanism to induce the placebo effect is learning. It has been demonstrated that different types of conditioning, such as classical conditioning or instrumental conditioning, are responsible for the formation of the

placebo effect (Benedetti et al., 2003; Colloca & Miller, 2011; de la Fuente-Fernández et al., 2009). Classical conditioning in the placebo field, has been proposed as the primary method to produce learning (Colloca et al., 2010; Montgomery & Kirsch, 1997; Voudouris et al., 1990). In this case, a previous experience of benefit associated with the exposure of a real treatment effect can later turn out to be beneficial when the real treatment is replaced by an inert (similar) treatment. The length of the learning process is a determinant factor for the placebo effect, in that a longer learning period during the placebo manipulation or treatment simulation resulted in stronger placebo response (Colloca et al., 2010). Interestingly, another type of learning, such as social observation, can produce placebo effects (Colloca & Benedetti, 2009; Schenk et al., 2017). Specifically, a person could learn how to respond to a condition by just observing the beneficial effect of a demonstrator after the application of a treatment.

These two-main cognitive mechanisms, expectation and learning, can interact to generate and maintain placebo effects (Ashar et al., 2017). In agreement with a cognitive interpretation of conditioning (Reiss, 1980; Rescorla, 1988), reinforced expectations can be induced after a conditioning procedure. In this way, after repeated exposure to the effects of a treatment, the individual knows what to expect when the treatment is applied again (Kirsch, 2018). Evidences converge in indicating that the placebo effect induced by the combination of both verbally-induced expectancy and conditioning results in stronger effects (Colloca et al. 2008; Schafer et al. 2015) than the placebo effect induced by the application of only verbal suggestion (Colloca et al., 2008; de Jong et al., 1996) or only conditioning (Montgomery & Kirsch 1997). Up to now, these evidences support the idea that recurrent positive experiences along with a cognitive ascription of benefit to the treatment is the best combination to obtain strong placebo effect (Ashar et al., 2017).

Neural correlates of placebo analgesia

Many studies have investigated the neural underpinnings of the placebo effect in pain. As we know, different cognitive mechanisms can induce a placebo response. Thus, the placebo effect cannot be supported by a simple brain mechanism or

system (Ashar et al., 2017). In Benedetti's words, *"there is not a single placebo effect but many"* (Benedetti, 2006). Therefore, different neural mechanisms could be also engaged depending on the function on which the placebo effect works (for instance, pain). Understanding the numerous neurobiological mechanisms that are involved in different placebo responses can help to understand the complex mind-brain-body interaction (Benedetti, 2006).

A considerable number of researches have explored the neural correlates of the placebo effect with different modern techniques such as electroencephalography (EEG), magnetoencephalography (MEG), functional magnetic resonance imaging (fMRI) or positron emission tomography (PET). It is important to note that most of the studies have focused on placebo analgesia (Ashar et al., 2017; Wager & Atlas, 2015). There is clear evidence that placebo procedures can significantly reduce pain-related responses occurring in the pain-processing system (Wager & Atlas, 2015). A clear and direct correlation was demonstrated between high placebo responses and reduced activity in pain-processing systems (Wager et al. 2007) or brain areas such as the dorsal anterior cingulate cortex, the thalamus and the mid- and anterior insula among others (Geuter et al., 2013; Wager & Atlas, 2015; Watson et al., 2009). For example, the responses related to pain that occur in the somatosensory areas and in the behavior are due to the network that connects the anterior cingulate cortex and the periaqueductal grey (Lui et al., 2010; Wager et al., 2004). Interestingly, the placebo effect is not related only with the central components of pain. Brain regions involved in higher-order cognitive functions, like anticipation of benefit and expectation play also a role in the placebo effect. Some of these brain areas show activation before and during painful stimulation, like the dlPFC, the ventromedial prefrontal cortex or the mid-lateral orbitofrontal cortex (Kong et al., 2006; Wager & Atlas, 2015; Watson et al., 2009). Moreover, the increased activity of the reported brain areas directly correlates with the amount of reported pain reduction (Wager & Atlas, 2015) (Figure 1).

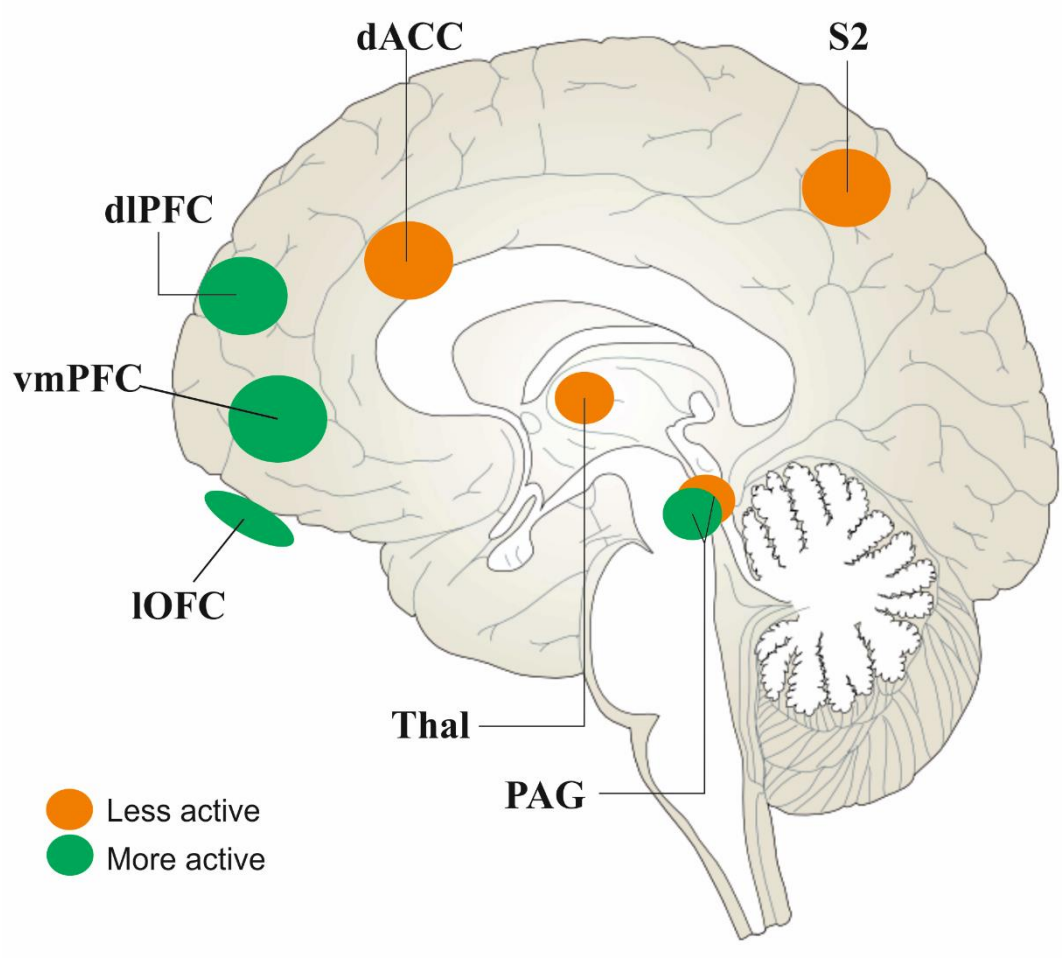

Figure 1. Neural correlates of placebo analgesia. A general view of some brain areas involved in the placebo analgesic effect. The areas that are represented in orange are involved in pain perception and show reduced activation after a placebo treatment. Some of these areas are the thalamus (Thal), periaqueductal grey (PAG), dorsal anterior cingulate cortex (dACC) and secondary somatosensory (S2) among others. Moreover, the areas that are represented in green are also related to other higher-order functions, like expectation or maintenance of context information. These areas show increased activation before or during a placebo treatment. These areas include the dorsolateral prefrontal cortex (dlPFC), the ventromedial prefrontal cortex (vmPFC), the lateral orbitofrontal cortex (lOFC) and periaqueductal grey (PAG) among others. *Adapted from Wager & Atlas, 2015*.

The placebo effect can also modify the release of different neurotransmitters. It has been discovered that the administration of naloxone, an opioid antagonist, could block the effect of placebo, thus demonstrating the involvement of the endogenous opioid system in placebo analgesia (Levine et al., 1978). From this first experiment, several studies have characterized the placebo effect in pain using naloxone, like reduction of heart rate or decrease β-adrenergic response (Colloca & Benedetti, 2005: Pollo et al., 2003).

The placebo effect in the motor domain

The investigation of the placebo effect in the motor domain is not new, going back to the 70's when Ariel and Saville (1972) performed a study on weightlifters and noticed that athletes who thought to have taken an ergogenic aid (actually a placebo) improved the performance. Since then, many other behavioral studies demonstrated the powerful effect of placebo in many sports (Beedie & Foad, 2009; Pollo et al., 2011). More recently, the investigation was enlarged to experimental models of motor performance in laboratory settings both in athletes and non-athletes (Benedetti et al., 2007; Carlino et al., 2014; Kalasountas et al., 2007; McKay et al., 2012). Finally, the interest on this field has become always wider to include also the neurophysiological investigation on the neural bases of the effect.
I will describe in more detail some of these studies in the following parts of my thesis, since my main interest was to study different levels of the placebo effect in the motor domain.

General aims

All the studies on placebo analgesia were very important in starting the experimental investigation on the neural and cognitive mechanisms of the placebo effect. As anticipated in the Preface, my interest was to export this investigation to the motor domain. In this regard, some questions still remain unanswered and during my Ph.D., I tried to design some studies to answer at least in part some of these questions. In particular, one goal of my research was to understand the role of a frontal brain area (like the dlPFC) in the placebo effect in the motor domain (*Part II*). Furthermore, I tried to enlarge the investigation of the behavioral aspects of the motor placebo effect by tackling two motor functions that are crucial in daily life activities (and not only in sports, as mainly investigated so far), like balance control and motor sequence learning (*Part III*).

PART II: NEURAL INVESTIGATION OF THE PLACEBO EFFECT IN THE MOTOR DOMAIN

Current knowledge on the neural correlates of the motor placebo effect

The neural correlates of the placebo effect in the motor domain are still largely unknown. So far, only two studies in healthy subjects have shown the contribution of some cortical brain areas in the placebo effect in the motor domain (Fiorio et al., 2014; Piedimonte et al., 2015). Fiorio et al. (2014) investigated whether the primary motor cortex (M1) could contribute to the behavioral increase of force production after a placebo procedure. To this end, the Authors used single-pulse transcranial magnetic stimulation (TMS) over M1 to evaluate the excitability of the corticospinal system. Motor evoked potentials (MEP) and cortical silent period (CSP) could be recorded with surface electrodes positioned over the muscle involved in the force task (i.e., the first dorsal interosseous). The amplitude of the MEP served as indirect measure of cortical and spinal motor circuits activity (Rösler & Magistris, 2008) and the duration of the CSP represented activity of inhibitory circuits at the cortical level (Wolters et al., 2008). Healthy subjects were randomized in four different groups: only verbal suggestion (I); verbal suggestion and conditioning (II); control with inert treatment (III) and control without inert treatment (IV). Participants were required to perform a motor task consisting of an abduction movement of the right index finger against a piston. They were instructed to press as strongly as possible and could see in real-time a visual feedback of the exerted force. Subjects of group I and II (experimental groups) underwent a placebo procedure consisting in the application of an inert electrical device (transcutaneous electrical nerve stimulation, TENS) over the muscle involved in the task together with positive verbal suggestion of improvement. Additionally, participants of group II received a conditioning procedure consisting of a surreptitious increase of the visual feedback of force. Groups III and IV served as control; with the former receiving the same TENS application but with overt verbal information that it was inert and the latter performing the same motor task but without TENS application. To explore the involvement of M1 in the placebo effect, single-pulse TMS was

delivered when all the participants exerted the same amount of force (i.e., 30% of the maximum voluntary force). Results showed higher MEP amplitudes after the placebo procedure in both experimental groups (I and II). Furthermore, the duration of the CSP was reduced only in the group who received verbal suggestion and conditioning (II). These findings suggest that the excitability of the corticospinal system can be modulated by a placebo procedure and results in higher force production.

The second evidence on the neural correlates of the motor placebo effect in healthy participants derives from an EEG study by Piedimonte et al. (2015). The study investigated the effect of a placebo procedure in reducing fatigue in a strength task. A specific component, called readiness potential (RP), was extracted from the EEG signal. The RP is associated to the preparation of voluntary movements (Shibasaki & Hallett, 2006) and is modulated by fatigue (Schillings et al., 2006; Slobounov et al., 2004). Moreover, the supplementary motor area (SMA) and M1 have been proposed as brain sources of the RP (Deecke, 1996; Shibasaki & Hallett, 2006). The task consisted of a repetition of flexion movements for lifting a weight with the index finger to induce fatigue. Participants were divided in two groups, placebo and control. The placebo group had to ingest a substance (actually inert) along with verbal suggestion that it was caffeine and could reduce fatigue. The control group, instead, did not received any treatment. Results showed that while in the control group there was higher perception of fatigue and higher RP amplitude after the procedure, in the placebo group perception of fatigue was reduced and the RP amplitude did not increase through the experiment. According to the Authors, a central mechanism could play a role in the placebo-induced decrease of fatigue, before movement execution (Piedimonte et al., 2015).

A different approach to investigate the neural correlates of the placebo effect in the motor domain derives from patients with motor symptoms, like Parkinson's disease. Parkinson's disease is a neurodegenerative disorder of the basal ganglia. A reduction of dopaminergic neurons in the substantia nigra is the cause of the occurrence of motor symptoms (Opara et al., 2017). A direct and straightforward comparison of the neural correlates of the motor placebo effect of parkinsonian patients with healthy individuals may be risky and a matter of bias. Nonetheless,

the results obtained from studies in parkinsonian patients can give important information on the neural correlates of the motor placebo effect. Parkinson's disease allows to explore how subcortical structures, like the basal ganglia, are related to cognitive and motor functions (Mallet et al., 2007).

Different studies have demonstrated that the dopaminergic system can be modulated by a placebo procedure. Specifically, de la Fuentes-Fernández et al. (2001 and 2002) made a positron emission tomography study to evaluate the amount of raclopride, an antagonist of dopamine receptors, after a placebo procedure in parkinsonian patients. The Authors found a reduced amount of raclopride in both the dorsal and the ventral striatum after placebo administration, suggesting that the placebo effect could be associated to the release of endogenous dopamine in subcortical structures (de la Fuentes-Fernández et al., 2001; de la Fuentes-Fernández et al., 2002). In another study (Lidstone et al., 2010), the amount of dopamine release in the striatum was modified depending on the told probability to receive an active treatment (actually a placebo). Precisely, patients who though to had 75% or 100% probabilities of receiving the treatment showed a significant increase of dopamine release, while those who though to had 25% or 50% of receiving the treatment did not show any change. The study demonstrated that the dopamine release in the striatum is related to the expectation of benefit subsequent to a placebo treatment (Lidstone et al., 2010).

The surgery to implant deep brain stimulation (DBS) in parkinsonian patients allows to evaluate the activity of neurons of the stimulated area. Electrophysiological studies showed the involvement of the subthalamic nucleus (STN) in the placebo effect. Benedetti et al. (2004) investigated the firing rate of the neurons of the STN after a placebo procedure in selected patients who were waiting for neurosurgical intervention with DBS. Once selected, patients underwent a conditioning procedure consisting of the administration of apomorphine (dopamine agonist) before the surgical intervention. During the surgery, injection of saline solution along with verbal suggestion of motor improvement was applied to the patients. The placebo procedure was applied during the electrophysiological recording of the neural activity in the STN. Benedetti et al. (2004) demonstrated that after a placebo procedure, some patients (responders) showed a reduction of

muscular rigidity and also a reduced discharge frequency and evoked non-bursting activity in the STN.

To deeper understand the potential changes of other subcortical structures in the motor placebo effect, Benedetti et al. (2009) recorded the activity of the substantia nigra pars reticulata (SNr), the ventral anterior (VA) and the anterior ventrolateral (VLa) nuclei of the thalamus. All these structures are involved in motor control and connected to the STN. Using a similar paradigm, they found a significantly lower activity in the STN and the SNr, whereas neuronal activity was higher in the VA and VLa nuclei only in patients who showed a reduced muscle rigidity. Moreover, a recent study demonstrated that to obtain clinical and neural changes (in the thalamus) a conditioning procedure should be used in which a real drug is administered together with the placebo procedure (Benedetti et al., 2016).

As we have been observing above, different cortical and subcortical structures (M1, SMA, STN, SNr, VA and VLa) are involved in the placebo effect in the motor domain and, interestingly, these structures are connected with other brain areas, such as the dlPFC (Hasan et al., 2013; Mayberg et al., 2002; Miller & Cohen, 2001), the cingulate cortex (Asemi et al., 2015; Mayberg et al., 2002; Petrovic et al., 2002), or the orbitofrontal cortex (Petrovic et al., 2002) which in turn have also been related to placebo analgesia (Ashar et al., 2017; Wager & Atlas, 2015). Hence, these evidences emphasize the idea that some of these areas could also have a role in the motor placebo effect. Among all these brain regions, the dlPFC seem to be a suitable candidate, due to it has a relevant role in placebo analgesia and in higher-order cognitive functions, like expectation and anticipation, which are considered the basis of the placebo effect. Remarkably, it has been demonstrated that it communicates with motor brain areas, thus indicating a potential role in the motor placebo effect.

In particular, this second part of my thesis describes a series of three experiments conducted to investigate the involvement of the dlPFC in the motor placebo effect. To this purpose, we proposed a motor task to measure the subjects' force level before and after a placebo procedure and modulated the activity of the dlPFC by means of non-invasive brain stimulation with transcranial direct current stimulation (tDCS). The selection of a force task among all the other motor task (i.e. movement

speed or resistance to fatigue) was due to one main reason. That is, a previous study has demonstrated the paradigm on the neural correlates of force during a placebo procedure (Fiorio et al., 2014). Thus, supporting by this study, we could investigate whether other brain areas involve in force (in our case the dlPFC) could also be modulated by a placebo procedure.

On the role of the dorsolateral prefrontal cortex

So far, a very limited number of studies attempted to investigate the potential neural correlates of the placebo effect in the motor domain. As mentioned previously, only two studies tackled this issue in healthy participants. More precisely, a first study has shown that the placebo-induced enhancement of force was related to an increase of activity in the left M1 (Fiorio et al., 2014). This was possible thanks to the application of TMS in healthy participants, which allowed to observe an enhanced amplitude of the motor evoked potentials and a shortening of the cortical silent period after the placebo procedure (Fiorio et al., 2014). A following EEG study showed that the placebo-induced decrease of fatigue was associated to a stable amplitude of the readiness potential (Piedimonte et al., 2015). The readiness potential is interpreted as the anticipatory phase of a movement and arises from the supplementary motor area and M1 (Deecke, 1996; Shibasaki & Hallett, 2006). Hence, the two studies in healthy participants indicate that two cortical areas (M1 and SMA), involved in movement execution and preparation play a role in the motor placebo effect.

Nonetheless, a more complex brain network is necessary for the cognitive control of motor behavior. The dlPFC together with other frontal regions, plays a prominent role in this complex network (Hasan et al., 2013; Miller & Cohen, 2001). It is worth mentioning that several studies have demonstrated that the dlPFC has an important role in placebo analgesia (Egorova et al., 2015; Geuter et al., 2013; Kong et al., 2006; Krummenacher et al., 2010; Lui et al., 2010; Peciña et al., 2013; Wager et al., 2004; Watson et al., 2009) and in elaborating expectation. As we know from the literature, expectation is one of the crucial cognitive mechanisms at the basis of the placebo effect (Krummenacher et al., 2010; Jubb & Bensing, 2013). Consequently, it is rational to conjecture that the dlPFC could be a potential actor in the placebo

modulation of motor performance. Our goal was to explore this hypothesis. To this end, we decided to apply the tDCS over the dlPFC during a placebo procedure in the motor domain, and precisely on a force task. The control of force requires the activation of both cortical and subcortical brain regions, like the prefrontal cortices, the cingulate motor area, premotor area, pre-SMA, SMA, the cerebellum and the basal ganglia, as we know from neuroimaging studies (Badoud et al., 2017; Dettmers et al., 1995; Ehrsson et al., 2000; Neely et al., 2013; Schmitz et al., 2005; Vaillancourt et al., 2007; Wasson et al., 2010). Several of these brain areas are connected with the dlPFC and they could exert a potential top-down control on force through the activity of the dlPFC (Alexander et al., 1986; Bates & Goldman-Rakic, 1993; Cieslik et al., 2013; Lu et al.,1994; Miller & Cohen, 2001; Schmahmann & Pandya, 1997; Petrides & Pandya, 1999). Additionally, the dlPFC is also involved in the selection of the quantity of force to be applied (Vaillancourt et al., 2007) and in the prediction of force amplitude (Wasson et al., 2010), supporting a direct role of the dlPFC in the cognitive control of force. As far as we know, no study so far has tackled the role of the dlPFC in the placebo effect on force.

Although there is clear evidence on the role of the dlPFC on placebo analgesia, there is not a definite role on the hemisphere (whether the left or the right dlPFC). Some studies reported that the left dlPFC is involved in placebo analgesia (Peciña et al., 2013; Watson et al., 2009), whilst other studies suggested the involvement of the right dlPFC (Egorova et al., 2015; Lui et al., 2010). Finally, other studies have demonstrated that the left and right dlPFC could act bilaterally in placebo analgesia (Kong et al., 2006; Krummenacher et al., 2010; Wager et al., 2004). It could be speculated that the type of experimental protocol and procedure adopted could partly explain the different results (Egorova et al., 2015; Lui et al., 2010). In our study, subjects had to perform the motor task with the right hand, requiring the involvement of the contralateral (left) primary motor cortex. Hence, in our study we hypothesized that the left dlPFC could be involved in the placebo-induced enhancement of force. The role of the left dlPFC was evaluated in three separate experiments and we implemented a within-subjects design in which anodal, cathodal and sham tDCS was applied to the same participant in three different days.

Transcranial direct current stimulation (tDCS)

We decided to apply non-invasive brain stimulation, in particular the tDCS, during a placebo procedure to investigate the left dlPFC. It may be worth describing the basic principles of tDCS before continuing with the study description. This little introduction about the tDCS helps to better clarify the reasoning behind the application of this technique together with a placebo procedure in the motor domain.

Non-invasive brain stimulation (NIBS) techniques, like repetitive transcranial magnetic stimulation (rTMS) and transcranial electrical stimulation (tES) are widely used as a research tool for studying the human motor and cognitive functions (Brunoni et al., 2012; Jacobson et al., 2012; Pascual-Leone et al., 1994; Perceval et al., 2016; Wassermann et al., 1998). Specifically, tES allows to modulate the state of the cerebral cortex by applying a very low electrical current over the scalp. Among all the existing types of tES, transcranial direct current stimulation (tDCS) has become the most widely known, investigated and used technique to investigate cognitive processes (Antal et al., 2014; Bestmann et al., 2015; Santarnecchi et al., 2015).

Back to the 60s, Bindman et al. (1964) demonstrated that the stimulation of the rat's cortex with subthreshold direct current induced a change of the cortical activity in a polarity-dependent manner (Bindman et al.,1964). Thirty years later, this effect was proven also in the human brain (Nitsche & Paulus, 2000; Priori et al., 1998). TDCS consists of a low-intensity direct current (1 to 2 mA) applied over the scalp by means of a pair of rubber electrodes (typically 25 or 35 cm^2) inserted in sponges that are soaked in saline solution (Stagg & Nitsche, 2011). TDCS can induce neural changes in the cortical activity, functional connectivity and metabolite concentrations and in this way, it can modulate human behavior (for a review, Fertonani & Miniussi, 2017; Nitsche et al., 2015). Thus, tDCS can temporarily interfere with the activity of a cortical network and in the meantime, it is possible to test the effects of this interface on specific tasks. Different motor and cognitive functions have been shown to be modulated by the application of tDCS on specific brain regions (for a review, Perceval et al., 2016; Shin et al., 2015).

Regarding the type of stimulation, tDCS can be applied in two active ways, anodal and cathodal stimulation. Concerning anodal stimulation, the anode (positive electrode) is placed over the target brain area under investigation whereas the cathode (negative electrode) is positioned over a reference area. With regards to cathodal stimulation, the electrode position is reversed, so that the cathode is placed over the target brain area of interest and the anode over the reference one. Furthermore, tDCS allows the application of an inactive condition, the so-called sham stimulation, which can emulate similar sensations on the skin like the active condition, but without any cortical modulation (Gandiga et al., 2006; Nitsche et al., 2008). Most of the studies indicate that anodal stimulation increases cortical excitability while cathodal reduces it. However, with regards to the cognitive domain, the inference anodal equals improved performance and cathodal equals impaired performance is not always possible. Namely, factors like the intensity of stimulation, the duration of stimulation or the cortical activation state at the time of the stimulation can produce different unexpected outcomes (Batsikadze et al., 2013; Monte-Silva et al., 2013; Silvanto et al., 2008). Thus, it is important to consider tDCS not only as a tool that can increase or decrease cortical excitability of a brain area but also a tool that can alter the signal to noise ratio in the stimulated area (Santarnecchi et al., 2015). Furthermore, authors like Bestmann et al. (2015) or Fertonani & Miniussi (2017) have aid to better understand the potential effects of tDCS considering different mechanisms inner to the method of stimulation.

The application of tDCS in the cognitive domain has allowed to investigate different goals, like the enhancement of cognitive functions, the investigation of the role of specific cortical areas involved in a specific function, and the investigation of the neurophysiological mechanisms related to a specific cognitive function (Parkin et al., 2015). Many studies have demonstrated the modulation of a considerable amount of cognitive functions with tDCS, like working memory (Schicktanz et al., 2015), strategic planning ability (Kaller et al., 2013), attention (Pecchinenda et al., 2015), semantic processing (Brückner & Kammer, 2017) or motor learning (Hardwick & Celnik, 2014). One of the crucial point of tDCS is the possibility to be applied together with motor or cognitive tasks, allowing thus to modulate a specific region not only before or after the performance of a task (offline

stimulation) but also during the performance of the task (online stimulation) (Thair et al., 2017)

Other features like being easily portable, having a low price and being easy to apply have made tDCS a widely used technique (Antal et al., 2014; Parkin et al., 2015; Sathappan et al., 2018). Furthermore, another advantage of tDCS is the very few side-effects that it evokes. An electrical stimulation with a current density between 0.028 to 0.08 mA/cm^2, usually induces a mild tingling or light itching sensation on the scalp during the stimulation, redness of the skin or more rarely, burning sensations (Nitsche et al., 2008; Poreisz et al., 2007). Thanks to this, tDCS produces very low side-effects compared to other NIBS techniques, like TMS. Last but not least, tDCS has a reliable sham condition that allows subjects to perceive the same sensation as during the active stimulation, without changing the cortical excitability (Gandiga et al., 2006; Nitsche et al., 2008). Taking altogether, tDCS can be considered as a potential tool to investigate the neural mechanisms of different cognitive and motor functions (Shin et al., 2015). Thus, tDCS is presented as a suitable method to investigate the selected brain area, like the dlPFC, during a placebo procedure in the motor domain.

Thus, as above mentioned, the role of the left dlPFC was evaluated in three separate experiments. We applied a within-subjects design in which anodal, cathodal and sham tDCS was applied to the same participant in three different days. In particular, in Experiment 1 (expectation alone) expectation was the main cognitive mechanism involved because the placebo procedure consisted of verbal suggestion alone. Instead, in Experiment 2 (expectation and conditioning) both expectation and learning were induced by the placebo procedure consisting of verbal suggestion and conditioning. Finally, in Experiment 3 (control procedure) the same force task was performed without any verbal suggestion and conditioning. This experiment allowed to rule out any effect of tDCS *per se* on motor performance. According to the general idea of tDCS in which anodal tDCS tends to induce excitability in the stimulated brain region, while cathodal tDCS induces inhibition (Filmer et al., 2014; Miniussi et al., 2013; Wagner et al., 2007), we anticipated that anodal and cathodal tDCS over the dlPFC should interfere with the motor placebo effect, by enhancing it or reducing it, respectively. On the other hand, sham tDCS is typically

applied as control stimulation and therefore it should not interfere with the placebo effect. Furthermore, we predicted that the effects of tDCS should arise especially in Experiment 1, in which expectation is the key mechanism attributable to the role of the dlPFC in expectation. Lastly, we anticipated that these effects could be more evident in placebo-responders, who may benefit more from the placebo procedure.

Methods

Participants

Three different experiments were carried out. The sample size was computed for each experiment using G-Power 3.1 (Faul et al., 2007), in which we considered F tests within factors with one group and six measurements (two sessions and three tDCS stimulations). We derived the effect size from a previous study that used the same motor placebo paradigm in different groups of healthy participants (Fiorio et al., 2014). For Experiment 1 (expectation alone), we used the information from the study of Fiorio et al., (2014). Precisely, in the group with expectation alone, the partial eta squared for the significant effect of Session (baseline vs. final) for the percentage of strong pressure ($Strong_{press}$), that represents force, was 0.111 equivalent to an effect size of 0.353. Regarding the obtained effect size (0.353), Power (1-β error probability) of 0.95, α error probability of 0.05, 6 measurements and correlation among repeated measures of 0, the resulting sample size is 28. Therefore, we made the decision to recruit more subjects to avoid dropping out of participants and to allow for a more robust counterbalancing of the stimulation sessions. Hence, 32 healthy volunteers were recruited (14 females; mean age: 21.4 ± 2.7). They were all right-handed expect one ambidextrous. In order to recruit participants with regards to Experiment 2 (expectation and conditioning), we based our sample size computation on a similar group from Fiorio et al. (2014) in which they received verbal suggestion and conditioning. The partial eta squared for the same interaction was 0.415, which correspond to an effect size of 0.842. Once again, by considering this effect size, α error probability of 0.05, Power (1-β error probability) of 0.95, 6 measurements and correlation among repeated measures of 0, the resulting sample size is 6. However, we made the decision to recruit more subjects to prevent drop-outs and to allow for a more robust counterbalancing of

the stimulation sessions. In this experiment, therefore, 19 healthy volunteers were recruited (8 females; mean age: 21.6 ± 2.9). They were all right-handed expect one left-handed. For the control group (Fiorio et al., 2014) the partial eta squared for the same interaction, described above, was 0.311, corresponding to an effect size of 0.671. Regarding the obtained effect size (0.671), α error probability of 0.05, Power (1-β error probability) of 0.95, 6 measurements and correlation among repeated measures of 0, the resulting sample size is 9. Thus, to prevent drop-outs and to allow for a more robust counterbalancing of the stimulation sessions 14 healthy volunteers were recruited (6 females; mean age: 23.5 ± 2.5). They were all right-handed expect one left-handed.

None of the participants presented neurological or psychiatric disease or contraindication to tDCS (Nitsche et al., 2008). Participants were free of medication (except contraceptives) at the time of the experiment. Moreover, participants were instructed to avoid consumption of alcohol and caffeinated drinks prior to the experiment. The protocol was approved by the local ethical committee of the Department of Neurosciences, Biomedicine and Movement Sciences of the University of Verona. Participants gave written informed consent in accordance with the Declaration of Helsinki and were debriefed about the placebo nature of the study only after completing the experimental procedure.

Motor task

To evaluate force, we selected a motor task consisting of pressing as strongly as possible a piston connected to a force transducer (DS BC302) with the right index finger (Fiorio et al., 2014). The finger pressures against the piston were linearly transformed in real-time into vertical displacements of a cursor visible on a PC monitor. In that way, subjects received a visual feedback on the level of force. The maximum voluntary force (MVF) was calculated for each single subject before starting the experimental procedure. By clicking a mouse with the left hand, subjects could decide when to initiate the trial. As soon as they started the trial, they had to press the piston as strongly as possible with the right index finger in order to move the cursor from a starting black line at the bottom of the screen into a coloured

target zone at the top of the screen. This target zone contained four lines that represented the 80%, 100%, 120% and 140% of the subject's MVF.
The motor task was performed in three sessions (described in detail below) and each consisted of 30 trials, lasting 1100 ms each. Subjects underwent the same procedure and motor task in three different days, in order to apply the three types of tDCS. The MVF calculated in the first day was used also for the other days.

Procedure

The first day of the experimental procedure, participants performed five trials to practice the motor task. In each experiment, the protocol included three sessions: baseline session, second session and final session (Figure 2). Subjects perform the same motor task in the baseline and in the final session. These two sessions were identical in the three experiments and allowed to evaluate the subjects' performance before and after the experimental procedure.
A placebo procedure was applied between the baseline and final sessions, in Experiment 1 and Experiment 2. Specifically, a 10Hz transcutaneous electrical nerve stimulation (TENS) was applied as an inert treatment for 3 minutes to the muscle involved in the task (the right first dorsal interosseous, FDI). When TENS was switched on, subjects felt a slight sensation on the skin without muscle contraction. Along with TENS application, the experimenter said that: "TENS is a new treatment used also in the clinical practice that has a direct effect in enhancing force production", according to Fiorio et al., 2014.
In Experiment 1 (expectation alone), participants went through a placebo procedure consisting of only verbal suggestion about the positive effects of TENS in enhancing force. In Experiment 2 (expectation and conditioning) participants underwent a verbal suggestion of positive benefits of TENS together with a conditioning procedure. In this case, unbeknown to the subjects, a surreptitious stepwise increase of the cursor's excursion range was introduced during the execution of the motor task. Precisely, the cursor's excursion was progressively augmented trial by trial in steps of 0.0105 from trial 1 to trial 20 and continued constant until the end of the session (from trial 21 to trial 30). The application of this conditioning procedure helped to strengthen the participants' belief in the

effects of TENS. Consequently, the two experiments were comparable, except for the presence or absence of a conditioning procedure.

Experiment 3 consisted of a control procedure in which subjects completed the same motor task in the three sessions, but in this case, the verbal information about TENS was different. Specifically, subjects were told that they belonged a control group and therefore TENS was applied with an inactive mode.

TENS was applied again before they started the final session. Then, subjects performed the motor task in the same way as in the baseline session (Figure 2).

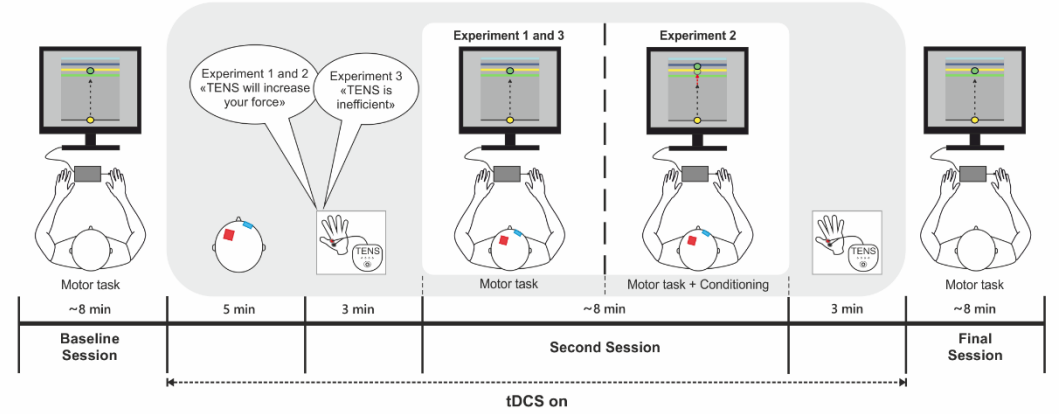

Figure 2. Schematic representation of the experimental protocol. The procedure consisted of three sessions (baseline, second session, and final). In each session, participants executed a force motor task by pressing a piston with the right index finger. A cursor on a PC monitor represented the visual feedback of force: the stronger the force exerted on the piston, the higher the excursion range of the cursor on the monitor. After the baseline session, electrodes for transcranial direct current stimulation (tDCS) were mounted according the stimulation day. The electrode montage represented in the figure refers to anodal tDCS; the position of the electrodes was reversed in the montage for cathodal tDCS. Sham tDCS had the same montage as anodal. tDCS was switched on and after 5 min the placebo (or control) procedure started. Transcutaneous electrical nerve stimulation (TENS) treatment was applied with different verbal information, according to the experiment. In the second session, the same motor task was performed again as in the baseline session (Experiment 1 and 3). In Experiment 2, instead, a conditioning procedure was applied with a surreptitious amplification of the visual feedback. tDCS was automatically switched off at the end of the procedure, for a total of 20 min of stimulation. After tDCS was switched off, participants performed the motor task in the same way as in the baseline (final session) *(Villa-Sánchez et al., 2018). With permission from John Wiley & Sons, Inc.*

Transcranial direct current stimulation (tDCS)

Anodal, cathodal and sham tDCS was applied in three different days for each experiment. The washout period between tDCS stimulation was by at least 72 hours. A battery-driven current stimulator (DC-Stimulator, BrainStim, E.M.S. Bologna, Italy) through a pair of 5 x 5 cm rubber electrodes was used to apply the tDCS. The electrodes were introduced into a sponge soaked with saline solution (0.09%) and were fixed using two rubber bands to the subject's head. The left dlPFC was stimulated due to the evidence resulting from studies on placebo analgesia (Peciña et al., 2014; Watson et al., 2009) and since the motor task was performed with the right hand, thus implying a major control of the contralateral left hemisphere. Furthermore, a previous study has shown that the left M1 is involved in the placebo effect using a similar task performed with the right hand (Fiorio et al., 2014). Therefore, as a result of the functional connectivity between the left dlPFC and the left M1 (Hasan et al., 2013), we decided to stimulate the left dlPFC. The F3-Fp2 electrode montage is commonly used to stimulate the left dlPFC (Tremblay et al., 2014; Wagner et al., 2007) and is suitable to modulate different functions of the dlPFC, like working memory, planning, executive functions (for a review, Tremblay et al., 2014).

Additionally, by using a computational model. We can observe that the propose setup is suitable to stimulate the left dlPFC (Figure 3). Hence, the electrodes were placed over F3 position, which has been consistently shown to approximate the scalp location overlying the dlPFC (Beam et al., 2009; Herwig et al., 2003; Mir-Moghtadaei et al., 2015; Rusjan et al., 2010;) and over Fp2, corresponding to the contralateral supraorbital area. TDCS polarity refers to the electrode over the left dlPFC (F3) and the montage was similar for anodal and sham tDCS (anode electrode over F3). HD-Explorer software (SoterixMedical, Inc., New York, NY) was used to check that the montage was suitable to stimulate the left dlPFC through simulation of the intensity of the current flow in the brain. As we can observe (see Figure 3), the proposed montage is able to reach the left dlPFC.

Furthermore, to place the electrode over the left dlPFC (F3) we used the Beam F3 System (Beam et al., 2009), which is based on the 10/20 EEG System and has a good approximation to MRI-guided neuronavigation (Mir-Moghtadaei et al., 2015).

A direct current of 1mA was applied (current density: 0.04 mA/cm^2) for 20 minutes with ramp up/ramp down of 10s during anodal and cathodal stimulation. Instead, for sham stimulation, the stimulation lasted for 30s at the beginning and at the end of the stimulation (ramp up/ramp down of 10s) maintaining the same intensity (1mA), while it was automatically turned off for the rest of the period.

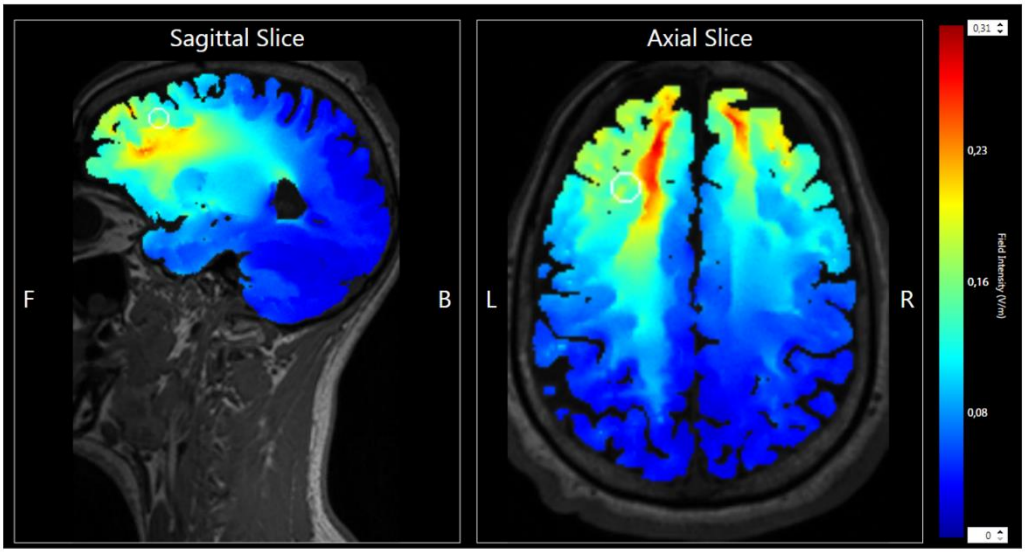

Figure 3. Computational model representation of the tDCS montage. Sagittal and Axial view of a head model's brain potentially stimulated by the tDCS over F3-Fp2 position (10/20 EEG System) at 1 mA. Dark red = the highest field intensity (V/m) and dark blue = the lowest field intensity (V/m). F = front view; B = back view, L = left view and R = right size.

The tDCS electrodes were mounted soon after the baseline session and removed before starting the final session, thus covering the placebo (or control) procedure. The stimulation began 5 minutes before the application of the TENS treatment along with the verbal suggestion and lasted for 20 minutes, until the beginning of the final session (Figure 2).

A sensation questionnaire related to tDCS was completed after each experimental day (Fertonani et al., 2015). Moreover, subjects were asked to judge whether they thought that tDCS was active or inactive, by answering a questionnaire where the answer "I do not know" was also considered (Fertonani et al., 2015).

A crossover and double-blind study was designed. All participants received, in counterbalance order, the three types of stimulation and both the experimenter and

the participant were unaware of the type of stimulation applied. Sham stimulation served as control and therefore each participant had his/her own control condition. The debriefing about the aim of the study and the type of stimulation occurred after the third day of experiments.

Measures of performance and perception

The main behavioural outcome of the study was force. Specifically, force was measured with two different indexes. First, the normalized force index consisted of the mean value of the force peak amplitude ($Force_{peak}$) calculated in the 30 trials of each session, normalized to the MVF. It was defined as follows:

$$\text{Normalized Force}_{\text{peak}} = \frac{\text{Force}_{\text{peak}}}{\text{MVF}} \times 100$$

The normalized force index represents the overall force in each session based on the initial MVF. The percentage of strong pressures ($Strong_{press}$) was the second index and it was measured for each session as follows:

$$\text{Strong}_{\text{press}} = \frac{\text{N}_{\text{strong trials}}}{\text{N}_{\text{tot trials}}} \times 100$$

where $N_{tot\ trials}$ is the total number of trials in each session (i.e., 30) and $N_{strong\ trials}$ is the number of trials in which the peak force amplitude was above the mean value computed in the baseline. The $Strong_{press}$ represents the number of times in which subjects pressed the piston above a certain value. Therefore, this index allows to know how constant were participants in maintaining force above a certain value throughout a session.

Subjective variables were also assessed throughout the procedure. More precisely, we measured the subjective perception of force, by asking participants to judge how strong they felt soon after the performance of the motor task on a 10 cm visual analogue scale (VAS) ranging from 0 (very weak) to 10 (very strong). In addition, subjective expectation about the effects of TENS was also measured right after each TENS application (before task performance). in this case, participants were asked

to answer how much and in which direction they expected the future performance would be compared to baseline on a 7-points NRS ranging from -3 (much worse than at baseline) to +3 (much better than at baseline), with 0 (the same as at baseline). Moreover, subjective perception of treatment efficacy was assessed after the execution of the motor task in the second and final sessions. Participants were told to judge whether TENS was effective or not in improving force production on a 10 cm VAS ranging from 0 (not effective at all) to 10 (extremely effective). Lastly, the sense of effort was measured after the execution of the motor task in each session using the Borg scale ranging from 6 (rest) to 20 (maximal effort) (Borg, 1970).

Statistical analysis

First level analysis – All participants

Outliers were removed prior to statistical analysis. More precisely, participants whose value in each variable and session was above or below the mean value of the group by 2.5 times the standard deviation of the group were considered outlier. By doing so, one outlier of Experiment 1 was eliminated regarding to normalized $Force_{peak}$. In each experiment, a first general analysis was carried out with repeated measures analysis of variance (rmANOVA) for the behavioural (normalized $Force_{peak}$ and $Strong_{press}$) and subjective parameters (perception of force, expectation, perception of treatment efficacy and sense of effort), with Stimulation (anodal, cathodal, sham) and Session (baseline, final) as within-subject factors. Moreover, one-sample t-test was used to compare the scores of expectation and treatment efficacy against 0, separately for the two applications (first and second) and for the three types of stimulation. This analysis allows to test whether participants were successfully suggested about the positive expectations of TENS and the perception of treatment efficacy.

Second level analysis – Placebo-responders

To better characterized whether active tDCS specifically modulates the motor placebo effect, a more fine-tuned analysis in Experiment 1 and 2 was conducted. In particular, we focused on participants who displayed a placebo effect, the so-called

«placebo-responders». Subjects who showed a consistent increase in the two indexes of force in the sham tDCS condition were defined as placebo-responders, assuming that the placebo procedure was the only experimental manipulation that could have affected motor performance due to the inactive nature of sham tDCS. Specifically, participants were qualitatively categorized as placebo-responders when the difference between the final and the baseline sessions in both normalized $Force_{peak}$ and $Strong_{press}$ in the sham condition was positive (Figure 5 and 7). We hypothesized that an increase of force during sham tDCS was not to be attributed to tDCS but to the placebo procedure, being sham tDCS inactive. Once participants were qualitatively categorized as responders, we then tested whether their performance was also quantitatively higher in the final compared to the baseline session. To this purpose, t-tests for paired samples were run to compare the final and the baseline session in both normalized $Force_{peak}$ and $Strong_{press}$ in the sham tDCS condition. Afterwards, we pursued our principal aim, which was to analyse whether active tDCS (anodal and cathodal) could modify the behavioural placebo effect in placebo-responders. A rmANOVA on normalized $Force_{peak}$ and $Strong_{press}$ was performed with Stimulation (anodal, cathodal) and Session (baseline, final) as within-subjects factors (the sham condition was excluded from this analysis, because it served to define placebo-responders). Additionally, the subjective variables (perception of force, expectation, perception of treatment efficacy and sense of effort) were analysed by means of rmANOVA with Stimulation (anodal, cathodal, sham) and Session (baseline, final) as within-subjects factors (in this case, the sham condition was included in the analysis, because the definition of subjects as responders was based on the behavioural variables).

For all the analyses, t-tests for paired samples were performed as post-hoc comparisons. The Bonferroni correction for multiple comparisons was applied where necessary. The level of significance was set at $p \leq 0.05$. Data are represented as mean ± SE.

Results

Experiment 1 – Expectation alone

First level of analysis – All participants

Higher normalized $Force_{peak}$ was found in the final (107.6 ± 1.6%) than in the baseline session (105.2 ± 1.2%) (factor Session, $F_{(1,30)}$= 6.97, p = 0.013) (Figure 4A). $Strong_{press}$ displayed similar results, with higher values in the final (59.3 ± 3.3%) than in the baseline session (50.4 ± 0.6%) (factor Session, $F_{(1,31)}$= 6.99, p = 0.013) (Figure 4B). This finding suggests that the increase of both indexes of force could be due to the expectation induced through verbal suggestion. Stimulation (for both indexes, p > 0.700) and the Stimulation × Session interaction (for both indexes, p > 0.670) were not significant. The analysis of perception of force showed a significant Stimulation × Session interaction ($F_{(2,62)}$= 4.15, p = 0.020), while Session (p = 0.110) and Stimulation (p = 0.130) were not significant. Post-hoc comparisons revealed that participants perceived to be stronger in the final (7.07 ± 0.23) than in the baseline (6.04 ± 0.30) session (p = 0.001) after sham tDCS, whilst no difference was found between the baseline and final sessions with both anodal and cathodal tDCS. Furthermore, participants perceived higher force after sham tDCS (7.07 ± 0.23) than after cathodal tDCS (6.31 ± 0.34) in the final session (p = 0.021) (Figure 4C).

The analysis of the expectation scores revealed that they were significantly above 0. This was true in both the first and the second application of TENS and in all the types of stimulation (for all comparisons, $t_{(31)}$ > 6.51, p < 0.001), indicating that positive expectations were induced by the procedure. Moreover, no difference between the first and second application (factor Session, p = 0.160) was found, suggesting that positive expectations continued to be stable throughout the procedure. Stimulation (p = 0.530) and Stimulation × Session interaction (p = 0.600) were not significant.

The analysis of perception of treatment efficacy revealed significantly different scores from 0, in both the first and the second application of TENS (for all comparisons, $t_{(31)}$ > 6.715, p < 0.001), indicating that subjects believed in the effects of TENS. Furthermore, no difference between the first and second application

(factor Session, p = 0.100) was found, suggesting that the perception of treatment efficacy remained stable throughout the procedure.

For the sense of effort parameter, no significant factors or interactions were found (for all effects, p > 0.460).

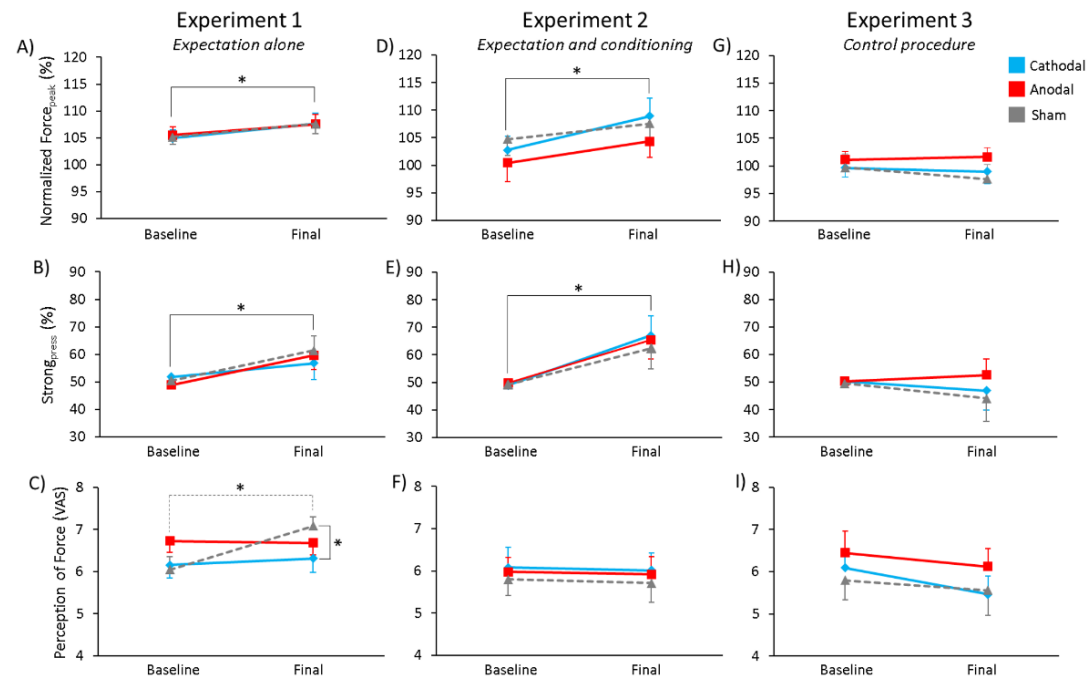

Figure 4. Behavioral and subjective data in the entire sample. (A) Normalized $Force_{peak}$ and (B) $Strong_{press}$ in Experiment 1 were higher in the final than in the baseline session, regardless of the type of stimulation. (C) Perception of force was higher in the final than in the baseline session only after sham tDCS, whereas it remained stable with anodal and cathodal tDCS. Moreover, a difference was found in the final session between cathodal and sham tDCS. (D) Normalized $Force_{peak}$ and (E) $Strong_{press}$ in Experiment 2 were higher in the final than in the baseline session, independently of the type of stimulation. (F) Perception of force did not change across sessions and type of stimulation. (G) Normalized $Force_{peak}$ and (H) $Strong_{press}$ in Experiment 3 (control), were stable across sessions and types of tDCS. (I) Perception of force did not change across sessions and type of stimulation. Cathodal, anodal and sham tDCS are represented with blue, red, and gray-dashed lines, respectively. Values are expressed as mean ± SE. *p < 0.050. *(Villa-Sánchez et al., 2018). With permission from John Wiley & Sons, Inc.*

Second level of analysis – Placebo-responders

Twenty out of 32 participants (62.5%) were defined as placebo-responders (9 women; mean age: 21.2 ± 2.3 years) due to a positive difference between the final and baseline session in the sham-tDCS condition, for both the behavioural indexes

(i.e., normalized $Force_{peak}$ and $Strong_{press}$, Figure 5A). The analysis comparing the mean values of force between the final and baseline session in the sham condition showed that placebo-responders had significantly higher force levels in the final (normalized $Force_{peak}$: 111.6 ± 2.2%; $Strong_{press}$: 79.7 ± 3.0%) than the baseline session (normalized $Force_{peak}$: 104.9 ± 2.0%; $Strong_{press}$: 50.5 ± 1.0%) (Figure 5B,C), thus confirming in a quantitative way the presence of a motor placebo effect when tDCS was inactive (i.e., in the sham condition) (normalized $Force_{peak}$: $t_{(19)} = -7.97$, $p < 0.001$; $Strong_{press}$: $t_{(19)} = -9.88$, $p < 0.001$).

The analysis of the effects of active tDCS in placebo-responders disclosed for normalized $Force_{peak}$ and for $Strong_{press}$ no effect of Session ($p > 0.078$), Stimulation ($p > 0.401$) or Stimulation × Session ($p > 0.150$) (Figure 6A,B). This finding indicates that the behavioural motor placebo effect found in the sham condition was absented when active tDCS was applied.

Perception of force revealed a significant Stimulation × Session interaction ($F_{(2,38)} = 3.62$, $p = 0.036$). Conversely, Session ($p = 0.178$) and Stimulation ($p = 0.680$) were not significant. Post-hoc comparisons disclosed that participants perceived higher force in the final (7.49 ± 0.30) than in the baseline (6.27 ± 0.37) session exclusively after sham tDCS ($p = 0.001$), but not after anodal and cathodal tDCS (for both comparisons, $p > 0.782$) (Figure 6C).

The analysis of expectation scores revealed that they were above 0 in both TENS applications and in all the types of stimulation (for all comparisons, $t_{(19)} > 6.11$, $p < 0.001$), indicating that placebo-responders expected an improvement in performance. Moreover, the expectation scores between the two TENS applications and between the different types of stimulation showed no differences (for all comparisons, $p > 0.138$).

The perception of treatment efficacy revealed a significant effect of Session ($F_{(1,19)} = 6.76$, $p = 0.018$), due to higher scores after the final (4.54 ± 0.59) than after the second session (4.05 ± 0.50). However, Stimulation ($p = 0.380$) and Stimulation × Session interaction ($p = 0.637$) were not significant. Furthermore, scores of treatment efficacy showed a significant difference from 0 both in the first and in the second application of TENS in all the types of stimulation (for all comparisons, $t_{(19)} > 5.86$, $p < 0.001$), indicating that participants believed in the effect of TENS.

The sense of effort revealed a significant Stimulation × Session interaction ($F_{(2,38)}$= 4.36, p = 0.020), but no effect of Session (p = 0.453) and Stimulation (p = 0.907). Post-hoc comparisons, however, were unsuccessful to disclose significant comparisons.

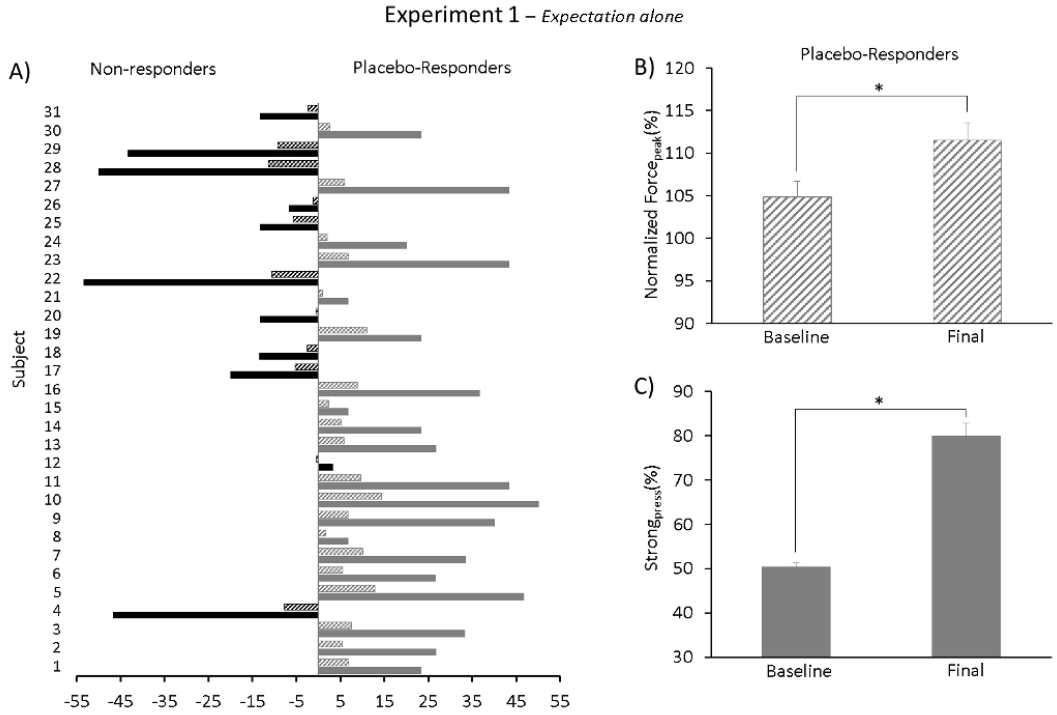

Figure 5. Number of placebo-reponders and non-responders of Experiment 1 (expectation alone). (A) Participants were categorized as placebo-responders when an increase in force (represented by a positive difference between the final and the baseline session) was consistently obtained for the two indexes of force, both normalized $Force_{peak}$ (striped-color bars) and $Strong_{press}$ (full-color bars) in the sham tDCS condition. (B) Normalized $Force_{peak}$ and (C) $Strong_{press}$ were analyzed to quantitatively confirm that in placebo-responders force was significantly higher in the final compared to the baseline session. Values are expressed as mean ± SE. *p < 0.050. *(Villa-Sánchez et al., 2018). With permission from John Wiley & Sons, Inc.*

Experiment 2 – Expectation and conditioning

First level of analysis – All participants

Higher normalized $Force_{peak}$ was found in the final (106.9 ± 2.8%) compared to the baseline session (102.6 ± 2.4%) (factor Session, $F_{(1,18)}$ = 8.50, p = 0.009). This suggests that the procedure was appropriate to induce a motor placebo effect (Figure 4D). Similar result was found for $Strong_{press}$, with higher values in the final (64.9 ±

4.3%) than in the baseline session (49.3 ± 0.5%) (factor Session, $F_{(1,18)}$= 12.61, p = 0.002) (Figure 4C). However, Stimulation (for both indexes, p > 0.350) and the interaction Stimulation × Session (for both indexes, p > 0.450) were not significant. For the analysis of perception of force, no significant factors or interactions was found (for all effects, p > 0.200) (Figure 4F).

The analysis of expectation scores was significantly above 0. This was true in both the first and the second application of TENS and in all the types of stimulation (for all comparisons, $t_{(18)}$ > 4.81, p < 0.001), indicating that positive expectations were induced by the procedure. Moreover, no difference between the first and second application (factor Session, p = 0.570) was found, suggesting that positive expectations continued to be stable throughout the procedure. Stimulation (p = 0.600) and Stimulation × Session interaction (p = 0.320) were not significant.

The analysis of perception of treatment efficacy revealed significantly different scores from 0, in both the first and the second application of TENS, indicating that subjects believed in the effect of TENS (for both comparisons, $t_{(18)}$ > 4.99, p < 0.001). Furthermore, Session was found significant ($F_{(1,18)}$= 30.65, p < 0.001), because of higher scores after the second session (4.54 ± 0.58) than after the final session (3.33 ± 0.58). Stimulation (p = 0.730) and Stimulation × Session interaction (p = 0.880) were not significant. For sense of effort, no significant results were found (for all effects, p > 0.200).

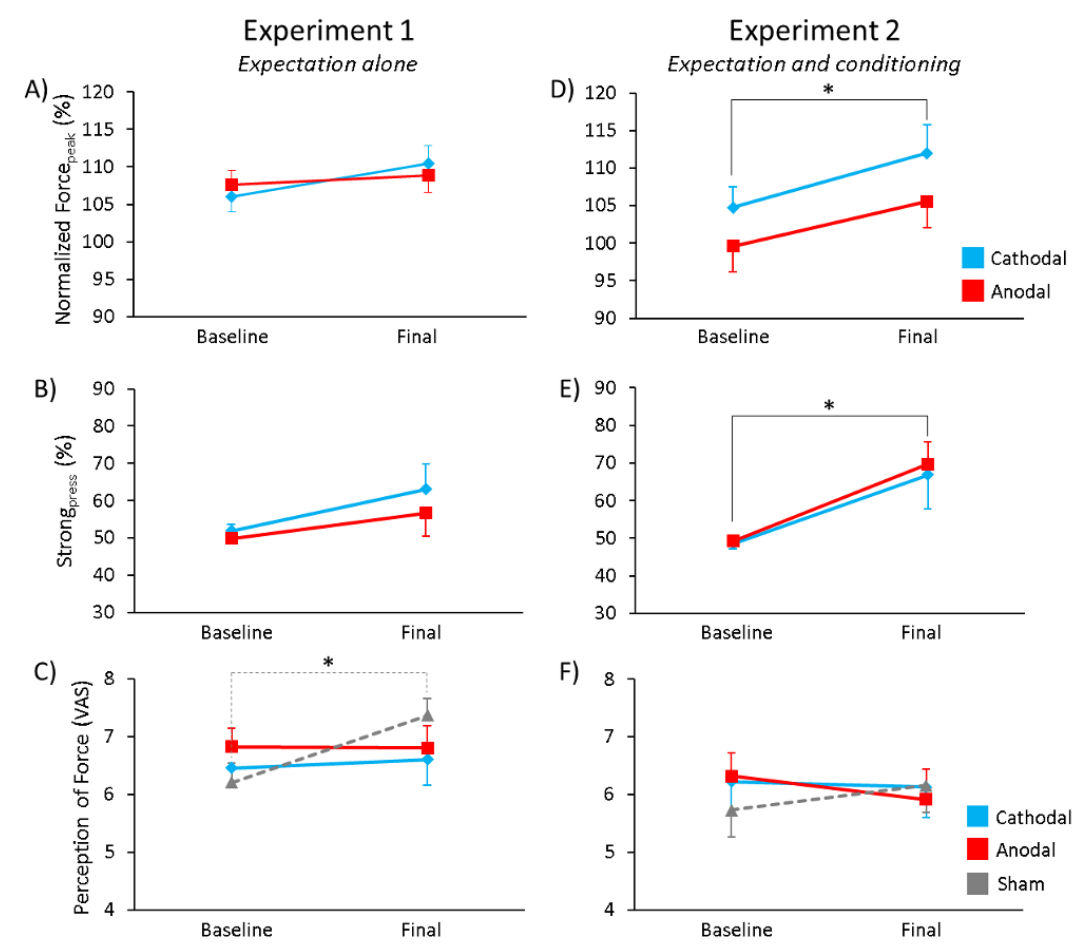

Figure 6. Behavioral and subjective data of placebo-responders. (A) Normalized Force$_{peak}$ and (B) Strong$_{press}$ in Experiment 1 remained stable across sessions, regardless of the type of stimulation. (C) Perception of force was higher in the final than in the baseline session only after sham tDCS, whereas it remained stable with anodal and cathodal tDCS. (D) Normalized Force$_{peak}$ and (E) Strong$_{press}$ in Experiment 2 were higher in the final than in the baseline session, independently of the type of stimulation. (F) Perception of force did not change across sessions and type of stimulation. Cathodal, anodal, and sham tDCS are represented with blue, red and gray-dashed lines, respectively. Values are expressed as mean ± *SE*.*$p < 0.050$. *(Villa-Sánchez et al., 2018). With permission from John Wiley & Sons, Inc.*

Second level of analysis – Placebo-responders

Fourteen out of 19 subjects (73.7%) were defined as placebo-responders (7 women; mean age: 21.8 ± 2.9 years) due to a positive difference between the final and baseline session in the sham-tDCS condition, for both the behavioural indexes (i.e., normalized Force$_{peak}$ and Strong$_{press}$, Figure 7A). The analysis of normalized

$Force_{peak}$ ($t_{(13)}$ = -5.31, $p < 0.001$) and $Strong_{press}$ ($t_{(13)}$ = -7.45, $p < 0.001$) in the sham condition confirmed that placebo-responders had significantly higher force levels in the final (normalized $Force_{peak}$: 111.3 ± 3.8%; $Strong_{press}$: 79.3 ± 4.4%) than in the baseline session (normalized $Force_{peak}$: 104.6 ± 3.4%; $Strong_{press}$: 49.5 ± 1.0%) (Figure 7B,C).

The analysis of the effects of active tDCS in placebo-responders disclosed for the normalized $Force_{peak}$ a significant effect of Session ($F_{(1,13)}$ = 10.51, p = 0.006), because of higher force levels in the final (108.8 ± 3.3%) than in the baseline session (102.2 ± 2.4%). Conversely, no effect of Stimulation (p = 0.129) or Stimulation × Session interaction (p = 0.720) was found (Figure 6D). Regarding the $Strong_{press}$, we also found a significant effect of Session ($F_{(1,13)}$= 12.52, p = 0.004), due to higher $Strong_{press}$ in the final (68.3 ± 5.2%) than in the baseline session (48.9 ± 0.9%), while Stimulation (p = 0.774) and Stimulation × Session interaction (p = 0.860) were not significant (Figure 6E). Therefore, unlike Experiment 1, active tDCS did not modify the motor placebo effect found in the sham condition, because the force levels were still higher in the final than in the baseline session, independently of the polarity of active tDCS.

Regarding the perception of force, no significant results were found in any factors or interactions (for all effects, $p > 0.100$) (Figure 6F). The analysis of expectation scores was again above 0. This was true for both TENS applications, indicating that responders expected an improvement in performance (for all comparisons, $t_{(13)} > 4.17$, $p < 0.010$). Moreover, the analysis between the two TENS applications and between the different types of stimulation was not significant (for all comparisons, $p > 0.200$). Treatment efficacy disclosed higher scores after the second session (4.71 ± 0.69) than after the final session (3.59 ± 0.72) (factor Session, $F_{(1,13)}$= 22.33, $p < 0.001$). Stimulation (p = 0.620) and Stimulation × Session interaction (p = 0.990) were not significant. For sense of effort, we did not find any significant factors or interactions (for all effects, $p > 0.100$).

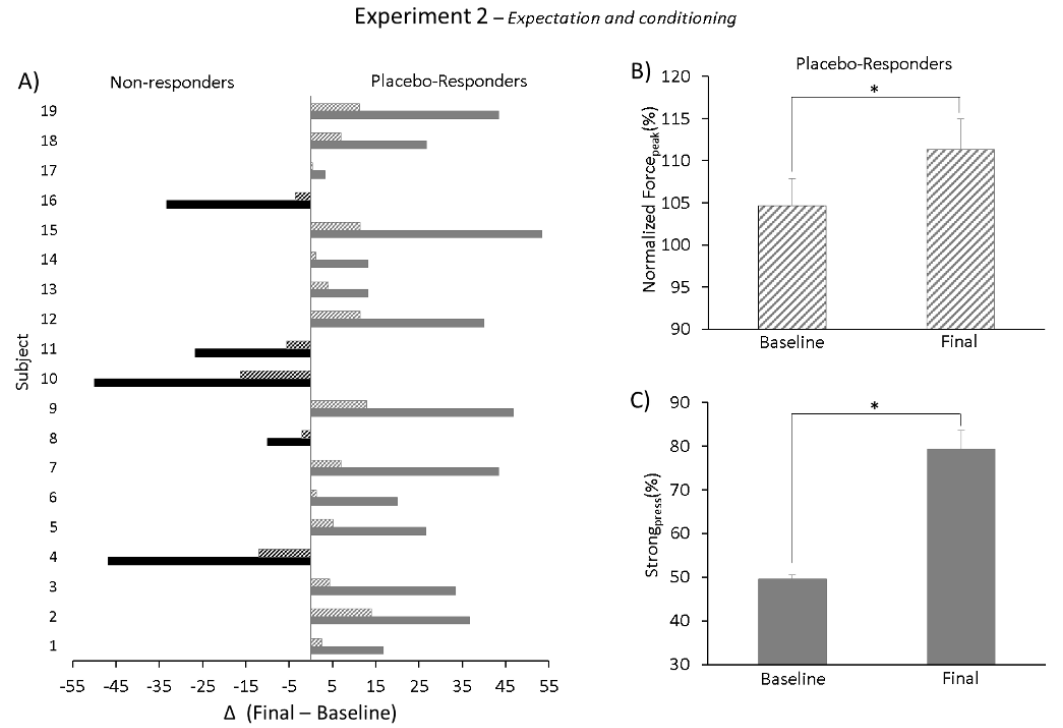

Figure 7. Number of placebo-reponders and non-responders of Experiment 2 (expectation and conditioning). (A) Participants were categorized as placebo-responders when an increase in force (represented by a positive difference between the final and the baseline session) was consistently obtained for the two indexes of force, both normalized $Force_{peak}$ (striped-color bars) and $Strong_{press}$ (full-color bars) in the sham tDCS condition. (B) Normalized $Force_{peak}$ and (C) $Strong_{press}$ were analyzed to quantitatively confirm that in placebo-responders force was significantly higher in the final compared to the baseline session. Values are expressed as mean ± SE. *p < 0.050. *(Villa-Sánchez et al., 2018). With permission from John Wiley & Sons, Inc.*

Experiment 3 – Control

Regarding the control procedure, no significant result was found for both the behavioural (for all the factors, $p > 0.390$) (Figure 4G,H) and for the subjective data (for all the factors, $p > 0.077$) (Figure 4I). These findings suggest that the control procedure did not induce any change in performance and that tDCS was unable to influence performance. We only found a significant effect in the analysis of expectation. In this case, session was significant ($F_{(1,13)}= 6.24$, $p = 0.027$) due to the lower values before the final (-0.01 ± 0.10) than before the second session (0.16 ± 0.09).

Discussion

The outcomes of this study demonstrate that an enhancement of force could be induced when a placebo procedure was adopted (for both Experiment 1 and 2) and not in the control experiment. Furthermore, the same amount of force before and after the placebo procedure was found specifically in placebo-responders who were only verbally suggested (Experiment 1) and received active tDCS (both cathodal and anodal). On the contrary, active tDCS did not influence the behavioural placebo effect when a conditioning procedure was used (Experiment 2). These results are discussed with regards to their significance for understanding the placebo effect in the motor domain and considering the differential involvement of the frontal brain regions (likely the dlPFC) in the placebo effect induced by either expectation alone or by combining both expectation and conditioning.

The placebo procedures induced motor placebo effects

In general, the behavioural outcomes of our study prove that higher levels of force could be induced comparing the final to the baseline session when a placebo procedure was applied with both verbal suggestion alone (Experiment 1) and with verbal suggestion plus conditioning (Experiment 2), in line with a previous study adopting a similar paradigm (Fiorio et al., 2014). Furthermore, the expectation scores were significantly above 0, suggesting the presence of positive expectations that continued to be stable throughout the sessions and days in both Experiment 1 and 2. Regarding the perception of treatment efficacy, our findings suggest that participants believed in the efficacy of TENS due to significantly above 0 scores. We found lower values following the second application of TENS compared to the first one in Experiment 2 and this could be due to the conditioning procedure. More precisely, in Experiment 2 the adoption of a conditioning procedure after the first TENS application might have induced subjects to perceive more effect soon after this application than the second one. In Experiment 3, no expectation was found, because the scores were not significantly different from 0. However, a lower expectation score at the end of the procedure was found and this could indicate that subjects expected a decrease of performance, likely due to mental fatigue. Regarding the subjective perception of force, an increase in the final compared to

the baseline session was found only in Experiment 1, while in Experiment 2 it remained stable, regardless of the behavioural increase of force. Recent studies have also described a dissociation between the objective and subjective components of the placebo effect (Rossettini et al., 2018; Schwarz & Buchel, 2015). Furthermore, a previous study using a similar paradigm have confirmed that the increase in subjective perception of force after a placebo procedure was qualitatively less marked in the group that received conditioning compared to the group with verbal suggestion alone (Fiorio et al., 2014). We might hypothesize that, as mentioned above, in Experiment 2 the conditioning procedure could have induced participants to perceive more force soon after the first application of TENS (when the visual feedback was surreptitiously amplified).

The role of the dlPFC on the motor placebo effect induced by expectation alone

At a first glance, independently of the type of tDCS stimulation, the force levels obtained in Experiment 1 were higher in the final compared to the baseline session, suggesting that a placebo effect could be induced with verbal suggestion alone and so forth, tDCS over the left dlPFC seemingly does not modulate this effect. Nonetheless, we found that when active tDCS was applied the force remained stable throughout the placebo procedure exclusively for placebo-responders. It is important to mention that these participants were selected for presenting a positive difference between the final and baseline session in both indexes of force when tDCS was inactive (sham tDCS). Moreover, a subsequent analysis verified that the levels of force in the final compared to the baseline session were significantly higher. Therefore, finding a constant motor performance in the same participants when active tDCS was applied indicates that the stimulation of the left dlPFC could have blocked a significant increase of force, otherwise present with inactive tDCS. Rationally, because the type of stimulation was counterbalanced across days we could rule out the possibility that these results are due to a regression to the mean. We think that active tDCS could have prevented the placebo-induced increase of force found in the sham condition. Additionally, we can exclude that this outcome was simply due to an effect of active tDCS on force production, considering that active tDCS *per se* did not disturb force in the whole sample of Experiment 1 and

Experiment 2. This block of force enhancement in placebo-responders could be attributed to a disturbance introduced by active tDCS in the brain network involved to the motor placebo effect.

Similarly, we found that the subjective findings were in line with the behavioural data. More precisely, we found higher perception of force after the placebo procedure exclusively in the sham tDCS condition and a stabilization with active tDCS. Nevertheless, differently from the behavioural data, the subjective results showed a stable perception of force with active tDCS when considering both the entire sample and just placebo-responders. Due to these findings, we could speculate that it could be easier to modulate the subjective component of to the placebo effect with active stimulation of the left dlPFC, whereas the objective component could be modulated by tDCS only in participants who present a clear placebo effect (placebo-responders).

We propose that our results could be interpreted by considering recent studies indicating that placebo effects induced by verbal suggestion alone preferentially involve prefrontal areas, like the dlPFC among others (Ashar et al., 2017; Wager & Atlas, 2015). However, this suggestion needs to be treated with caution. It has been found that the dlPFC is an associative area related to several higher-order cognitive functions, such as working memory (Schicktanz et al., 2015; Shen et al., 2015), strategic planning ability (Kaller et al., 2013) and attention (Pecchinenda et al., 2015). Furthermore, some studies have shown that the dlPFC is decisive for placebo analgesia (Egorova et al., 2015; Geuter et al., 2013; Kong et al., 2006; Krummenacher et al., 2010; Lorenz et al., 2003; Lui et al., 2010; Peciña et al., 2013; Wager et al., 2004; Watson et al. 2009). It is worth mentioning, that the dlPFC has many structural and functional connections with several brain areas related to motor control, like the premotor areas, the supplementary motor area, the basal ganglia and the M1 (Alexander et al., 1986; Hasan et al., 2013; Lu et al., 1994; Miller & Cohen, 2001), and moreover, this structure (the dlPFC) has a major function in the executive top-down control of behaviour (Miller & Cohen, 2001). Considering the context of placebo effect in the motor domain, all these connections could have a relevant role in its generation or modulation.

Additionally, previous studies have demonstrated that the dlPFC is implicated in the selection of the amount of force (Vaillancourt et al., 2007) and in the prediction of force amplitude (Wasson et al., 2010), thus indicating that the dlPFC is involved in the cognitive control of force. Therefore, due to the fact that the dlPFC has several connections with brain areas involved in controlling motor behaviour and force and is involved in expectation, which is the main cognitive mechanism induced by verbal suggestion, we could suggest that when only verbal suggestion is adopted, the dlPFC could play a particular function in the placebo-induced modulation of force. This interpretation is supported by Krummenacher et al. (2010), in which the application of low-frequency repetitive TMS (that has an inhibitory effect) over the dlPFC blocked placebo analgesia induced by verbal suggestion alone.

The role of the dlPFC on the motor placebo effect induced by expectation and conditioning

With regards to Experiment 2, we saw that active tDCS on the dlPFC did not modify the behavioural increase of force. In this experiment, both verbal suggestion and a conditioning procedure were applied. Hence, we could suggest that other brain areas could be involved when a conditioning procedure is adopted, apart from the dlPFC. Of note, the slightly amplified visual feedback introduced in the conditioning session, could have acted as a positive reinforcement. It has been shown that the application of a positive visual feedback can enhance motor performance, and this process relies on reward mechanisms (Lutz et al., 2012). Therefore, we could speculate that the conditioning procedure in Experiment 2 induced the engagement of brain areas related to the reward circuitry, like subcortical structures (de la Fuente-Fernández et al., 2002; de la Fuente-Fernández, 2009; Enck et al., 2008; Lutz et al., 2012). The placebo effect obtained with this procedure could have been more consolidated than with verbal suggestion alone (Experiment 1) and thus less subject to the exclusive influence of the dlPFC. This interpretation, however, remains speculative.

Alternatively, we could also suggest that the right dlPFC, more than the left, could be critical in the placebo effect when a conditioning procedure is applied. Some

evidence indicates that the fronto-parietal network of the right hemisphere, including the right dlPFC, may be involved in conditioning (Egorova et al., 2015; Kong et al., 2013; Lui et al., 2010), giving a support to our hypothesis. The study done by Egorova et al. (2015) showed that the placebo effect induced using a cue conditioning paradigm without verbal suggestion was modulated by anodal tDCS over the right dlPFC. According to the authors' interpretation, the learned associations during the cue conditioning could have been consolidate with anodal tDCS, thus enhancing the placebo effect. However, we need to consider some methodological differences between our study and that by Egorova et al. (2015). Whereas in our study we used online stimulation inserted in the placebo procedure itself, the study by Egorova et al. (2015) used offline stimulation (after the placebo procedure). Because online and offline tDCS stimulation could produce different effects, we speculate that the discrepancy between our studies could be described by this methodological choice. Furthermore, it could be considered that the effects of tDCS or the involvement of the dlPFC could be different since the placebo effect in our study was in the motor domain and not in pain. Despite these differences, the likelihood remains on the apparently differential role of the left and right dlPFC in the placebo effect elicited by expectation alone or by a conditioning procedure. Together with this reasoning, we could also speculate that eliciting a placebo effect through verbal suggestion alone may engage more the left hemisphere than the right one due to the fact that the left hemisphere is usually related to linguistic functions. According to this hypothesis, we could explain why in the experiment in which verbal suggestion was the only method to induce the placebo effect (i.e., Experiment 1) an active stimulation over the left hemisphere produced an effect on the placebo response, and particularly in participants who seemed to be more suggested by the experimenter's words (i.e., placebo-responders).

Polarity-independent effects of tDCS

Our findings indicate that both cathodal and anodal tDCS induced the same results in placebo-responders. Thus, differently from our predictions, we did not find a polarity effect of tDCS. Similar to our findings, some studies have shown that both anodal and cathodal tDCS can produce the same behavioural and

neurophysiological outcomes, thus suggesting a polarity-independent effect (Antal et al., 2007; Batsikadze et al., 2013; Stagg et al., 2013). Stagg et al. (2011) applied anodal, cathodal and sham tDCS before an explicit motor sequence learning task. The Authors found that the reaction times in the motor task after both anodal and cathodal tDCS were longer compared to sham tDCS. Moreover, the application of anodal or cathodal tDCS during a motor exercise resulted in lower MEPs amplitude than during a resting condition (Antal et al., 2007). Additionally, a study carried out by Batsikadze et al. (2013) found that both anodal and cathodal tDCS over the left M1 induced similar MEPs amplitude.

Concerning our findings, we could speculate that tDCS, independently of the polarity, might have perturbed the proper functioning of brain areas that are selectively engaged and activated in a specific task. According to Silvanto et al. (2008), the effect of brain stimulation can be different according to the cortical activation state at the time of stimulation. In other words, the effects that the electrical current (an external stimulus) induces on a brain area are influenced by the physical properties of the stimulus and by the baseline activation state of that region. This effect has been called state-dependency. Therefore, a better interpretation of our finding could be made by considering the interaction between a brain state induced with the placebo procedure and the electrical modulation of a brain area potentially involved. Thus, our outcome can be interpreted according to the stochastic-resonance model. According to this model, an interaction occurs between the electrical current introduced into a system and the activity of this system (Fertonani et al., 2017; Miniussi et al., 2013; Kitajo et al., 2003). Referred to our procedure, we might speculate that tDCS inserted an input (noise) in a brain network that was functioning correctly (as it is supposed to be in placebo-responders), thus consequently perturbing the usual activity of that network. This could explain why both anodal and cathodal tDCS abolished the induction of the motor placebo effect. As mentioned above, this outcome arose just in Experiment 1, in which the main cognitive mechanism involved was expectation, and hence a crucial role of the left dlPFC was more likely.

Potential limitations of the study

Despite the advantages of tDCS (e.g., few side effects; reliable sham condition), it should be recognized some points of weakness, such as for example the low spatial resolution, due to the fact that the electrical current can spread and affect other brain areas (Datta et al., 2009). Therefore, it is difficult to rule out the fact that tDCS could have stimulated other brain structures functionally active in our task and belonging to a network involved in the motor placebo effect. However, we might assume a major contribution of the left dlPFC to our findings, considering the electrode montage (F3-Fp2) and the unimanual motor task (right hand) applied in our study.

Regarding the electrode montage, some evidence agrees with the idea that F3-Fp2 montage allows a suitable current magnitude underneath the anode electrode placed on F3, which corresponds to the left dlPFC (Wagner et al., 2007). With regards to cathodal stimulation, similar current density distribution should be found underneath the cathode electrode over F3 (Miranda et al., 2006). Furthermore, it has been shown that the F3-Fp2 montage can properly modulate different cognitive functions of the dlPFC, like working memory, planning and executive functions (for a review Tremblay et al., 2014). For this reason, we believe that the electrode montage used in our study was appropriate for targeting the left dlPFC.

Of course, the electrical current could also affect the activity of the brain area underneath the electrode located on Fp2. Moreover, Fp2 seems to correspond to the right orbitofrontal cortex (Koessler et al., 2009; Nejati et al., 2018; Willis et al., 2015), which is a brain area known to be involved in placebo analgesia (Wager et al., 2011; Wager & Atlas, 2015). Nonetheless, we think that this area may have a weak significant contribution to our findings because the electrode was located on the hemisphere ipsilateral to the hand executing the motor task (i.e., the right). Specifically, a unimanual task made with the right hand principally activates the contralateral left hemisphere and hence, we can better explain our finding by considering the effects of the current on the brain area under the F3 electrode (i.e., the left dlPFC). It is worth mentioning that the dlPFC has been shown to be predominantly active on the side contralateral to the used hand (Ehrsson et al., 2000; Ehrsson et al., 2001; Neely et al., 2013; Wasson et al., 2010). In addition, the left

dlPFC has functional connections with the left M1 (Hasan et al., 2013) and a previous study has demonstrated that the left M1 is involved in the placebo-induced enhancement of force (Fiorio et al., 2014). Furthermore, there is evidence showing that a dynamic force task (as we used in our study) requires a stronger participation of the left hemisphere (Neely et al., 2013). Therefore, all these reasons indicate that the brain area mainly affected by the electrical current and modulating our findings was the area located in the left hemisphere and corresponding to the position of the F3 electrode (i.e., the left dlPFC).

Finally, to better clarify the precise involvement of the dlPFC in the motor placebo effect in future studies, it could be useful to combine our experimental protocol with neurophysiological (TMS) and neuroimaging (fMRI) techniques. This could aid to clarify the contribution not only of the dlPFC but also of other brain areas in the placebo effect in the motor domain.

PART III: ENLARGING THE BEHAVIORAL INVESTIGATION OF THE PLACEBO EFFECT IN THE MOTOR DOMAIN

Current knowledge on the behavioral aspects of the motor placebo effect

Most of the investigations conducted to understand the placebo effect, have focused on pain. Nonetheless, since placebo effects are the results of a complex interaction between a specific organism and its surrounded environment, it is reasonable to find placebo effects beyond the healing context (Pollo et al., 2011). With regard to this, several behavioral studies have demonstrated that the placebo effect can influence also motor performance (Beedie & Foad, 2009; Pollo et al., 2011).

Compared to the study of the placebo effect in pain, knowledge is much less advanced in the motor domain. Nonetheless, several studies have demonstrated that the use of a placebo procedure can improve force production (Bottoms et al., 2014; Fiorio et al., 2014; Kalasountas et al., 2007; Maganaris et al., 2000; Pollo et al., 2008). Maganaris et al. (2000) showed that the administration of a placebo pill (saccharine) along with the information of receiving an anabolic steroid could increase force production during a weight lifting task in a sample of professional weightlifters. A study by Kalasountas et al. (2007) found that healthy participants who were verbally induced to expect higher force production after the ingestion of a fake performance-enhancing substance, showed higher force levels than subjects of the control group.

Movement speed is another dimension of motor performance that can be boosted with placebo procedures. This has been mainly demonstrated in sports like cycling and running (Beedie et al., 2006; Beedie et al., 2007; Foster et al., 2004; McClung & Collins, 2007; Porcari et al., 2006). For instance, Beedie et al. (2006) investigated how a placebo procedure could improve the performance of a group of well-trained cyclists in a 10-km race. Subjects, at first, were given information about the efficacy of caffeine in improving speed. They underwent three different conditions in which they were told to drink an inert substance and two caffeine-based drinks with low and high dose of caffeine (all were actually placebos). Higher performance was found after the cyclists thought to have drunk the caffeine-based drinks compared

to the inert substance. Moreover, Beedie et al. (2006) demonstrated a dose-dependent effect of the placebo, since cyclists were twice as fast when they thought to have drunk the high dose caffeine drink compared to the low dose drink. In another study, a group of experienced runners who thought to have ingested an ergogenic aid (actually a placebo) reduced their time (increase of speed) in a 5-km time trial compared to the condition in which they knew to have ingested water (control) (Porcari et al., 2006).

Furthermore, many studies have shown improvement of resistance to fatigue as a consequence of the induction of the placebo effect (Benedetti et al., 2007; Carlino et al., 2014; Carlino et al., 2016; Clark et al., 2000; Piedimonte et al., 2015; Pollo et al., 2008). One of the first studies showing the beneficial effect of expectation in reducing motor fatigue was the study by Clark et al. (2000). A group of athletes was assessed in a 40-km cycling race. Subjects were randomized in three groups with different information: one group was told to receive a carbohydrate substance (group 1), one group was told to receive a non-caloric sweetener (group 2), and one group was told to have 50% chances of receiving carbohydrate (group 3). Unbeknown to the participants, though, each single group was actually randomized in two subgroups: 50% of the participants received carbohydrate and the other 50% a placebo. The subject who were told to receive carbohydrate (group 1) showed an increase in power compared to the other two groups. Interestingly, the performance was higher in the subgroup who received a placebo rather than real carbohydrate (Clark et al., 2000).

The improvement of a specific motor performance through a placebo procedure has been confirmed in both athletes (Beedie et al., 2006; Beedie et al., 2007; Benedetti et al., 2007; Carlino et al., 2014; Clark et al., 2000; Maganaris et al., 2000; McClung & Collins, 2007; Porcari et al., 2006) and non-athletes (Bottoms et al., 2014; Carlino et al., 2016; Fiorio et al., 2014; Kalasountas et al., 2007; McKay et al., 2012; Piedimonte et al., 2015; Pollo et al., 2008). Indicating the capacity of placebos to enhance a motor performance independently of the skill level previously develop. It is worth noting that also in the motor domain, the placebo effect is related to the two cognitive mechanisms proposed for placebo analgesia, that is expectation (Beedie et al., 2006) and learning (Fiorio et al., 2018; Pollo et al., 2008).

Correspondingly, it is proven that the best method to induce robust and strong placebo effect in the motor domain is the application of both verbally-induced expectation and conditioning (Fiorio et al., 2014; Fiorio et al., 2018; Pollo et al., 2008). Therefore, the formation of the placebo effect through the combination of these two mechanisms is in accordance with the knowledge obtained in placebo analgesia studies (Ashar et al., 2017; Colloca et al. 2008; Vase et al., 2002).

Even though the growing interest on the placebo effect in the motor domain, some questions remain open. So far, the behavioral evidence has shown that the placebo effect is capable to modulate some dimensions of motor performance such as force production, movement speed and resistance to fatigue (Beedie & Foad, 2009; Fiorio et al., 2018; Pollo et al., 2011). However, the potential effects on other critical parameters of motor performance remains unknown. That is to say, motor performance is a multifaceted concept that embraces different dimensions, not only movement speed, resistance to fatigue and force, but also precision control, balance, visuomotor coordination, motor sequence learning and motor adaptation, among other. Some of these motor functions are important not only in sports but also in daily life activities. Therefore, enlarging the behavioral investigation of the placebo effect on other motor functions may help to achieve a better understanding of the placebo effect itself as well as to expand its range of application to daily life activities. To this end, we tried to explore whether a placebo procedure can improve two relevant motor functions present in our daily life, like balance control and motor sequence learning.

Balance can be defined as the ability to maintain the body in equilibrium (O'Sullivan, 2007). Balance control is present in every activity of the daily life. It also allows to maintain stable position preventing, therefore, falls. On the contrary, an impairment of balance control can provoke serious disturbances, limiting the quality of life (Maki et al., 1994; Pfortmueller et al., 2014). As an example, gait disorders such as those present in Parkinson's disease or in the elderly population lead to higher risk of fall, which limits the quality of life. Hence, extending the potential beneficial effects of placebos on balance control may allow to provide new strategies for the rehabilitation of gait disorders for which the pharmacological treatment is often not effective.

Briefly, we can define motor skill learning as an important motor function that permits to convert isolated and specific motor movements into well-performed skills through practice (Dayan & Cohen, 2011; Wolpert et al., 2011). Learning motor sequences is very important for many tasks such as cooking or open a door, and to develop well-executed motor skills like writing or cycling that are present during the lifespan. Furthermore, the possibility to improve this function is relevant in rehabilitative training after an injury (i.e. stroke) (Kitago & Krakauer, 2013).

Interestingly, both balance control and motor skill learning rely on complex neural networks connecting several cortical regions (Doyon et al., 2003; Doyon et al., 2009; King et al., 2013; Krakauer & Mazzoni, 2011; Obata et al., 2009; Penhune & Steele, 2012; Tokuno et al., 2009). Remarkably, some of these cortical regions (like the primary motor cortex M1, the supplementary motor area SMA, the premotor cortex PM) overlap with the brain areas involved in the placebo effect in the motor domain (Fiorio et al., 2014; Piedimonte et al., 2015). These evidences help to hypothetically link these two components of motor performance with the placebo procedure.

This third part of my thesis, is divided in two main behavioral studies in which we attempted to fill in the knowledge of gap about the behavioral evidences of placebo effect the motor domain. To achieve this goal, we conducted a first study, in which we aimed at investigating whether positive expectations induced through verbal suggestion can modulate the control of balance, and a second study, in which we explored whether the application of a placebo treatment consisting of verbal suggestion could help in improving motor sequence learning. In this case, we also aimed to tackle a differential role of two types of placebo treatments: one motor and one cognitive.

The placebo effect on balance control

As aforementioned, many behavioral studies have demonstrated the efficacy of placebos in influencing aspects of motor performance like speed, force, and resistance to fatigue in athletes and non-athletes alike (Beedie et al., 2006; Beedie & Foad, 2009; Fiorio et al., 2014; Piedimonte et al., 2015; Pollo et al., 2011). Motor performance, however, is a multifaceted definition that refers to several different

dimensions. As we know, the potential effect of placebos on different aspects of motor performance is still lacking. In the present study, we aimed at investigating whether the placebo effect can influence a very important component of human motor functions, like balance control.

Balance can be defined as the capacity *"to control the body's position in space for stability and orientation*" (O'Sullivan, 2007). It allows us to keep a stable and upright stance needed for many daily life activities such as getting up from bed, walking, waiting on a queue or simply taking a shower, and also for preventing fall. Human stance is not a static but a dynamic phenomenon (Boyas et al., 2013; Gunther et al., 2009), characterized by small instabilities periods called body sways. Controlling posture through balance requires a complex organization and interaction between motor coordination and sensory systems, such as the somatosensory, vestibular and visual systems (Horak, 1997; Perteka & Loughlin, 2004) and other brain regions.

In particular, the cerebral cortex has a significant role for human balance control (Jacobs & Horak, 2007a; Papegaaij et al., 2014; Wittenber et al., 2017), as confirmed using cortico-muscular coherence (Murnaghan et al., 2014; Vecchio et al., 2008), electroencephalography (Mierau et al., 2017; Slobounov et al., 2008) and neuroimaging (Ouchi et al., 1999) studies. Furthermore, TMS studies demonstrated enhanced corticospinal excitability in standing balance compared to non-standing or supported conditions, suggesting a role of the corticospinal system in this function (Obata et al., 2009; Tokuno et al., 2009). Critically, the placebo effect in the motor domain seems to increase the excitability of the corticospinal system (Fiorio et al., 2014), thus giving neurophysiological support to the connection between the placebo effect and balance control.

In the present study, we used a parallel design in which a single-leg stance task was performed by two groups of healthy participants (placebo vs. control). The placebo group received the application of an inert electrical device over the leg muscle together with verbal information about its positive effects on balance. The control group received the application of the same device with overt information about its inefficacy in changing balance. To measure balance, we developed a new custom-made user-friendly device consisting of a sensor fixed in the leg that easily permits

a fine-tuned detection of body sways in different directional planes, without any platform or heavy equipment. This feature makes it practically useful in future studies aiming at exporting this paradigm outside the laboratory to improve balance control in patients affected by postural deficits or in gait disorders in which the pharmacological treatment is often ineffective. We hypothesized that subjects of the placebo group would enhance balance control after the procedure compared to the control group.

Methods

Participants

Thirty healthy participants were recruited (14 females; mean ± SD: 20.1 ± 1.3 years) from the student population of the University of Verona. Participants were separated in two different groups matched for sex, age, height and foot size: 15 subjects (7 females; mean age: 20 ± 1.4 years; mean height: 172.3 ± 10.8 cm; mean foot size: 26.5 ± 1.9 cm) were enrolled in the placebo group, and 15 subjects (7 females; mean age: 20.1 ± 1.1 years; mean height: 171.4 ± 8.8 cm; mean foot size: 26.3 ± 1.8 cm) were enrolled in the control group. Since balance is affected by the base of support (foot measure) and the distance of this to the centre of mass (Height). We decided to control and matched height and foot size to exclude any bias in the balance motor task. Participants were all dominant on the right lower limb, according to the Edinburgh questionnaire (Oldfield, 1971). Participants gave their written informed consent at the beginning of the experiment and were debriefed about the placebo nature of the study only after completing the whole experimental procedure. The study was approved by the committee for approval of research on humans (CARU) of the University of Verona.

Single-leg stance task

The task consisted of standing on the floor as steadily as possible with the dominant leg for 30 seconds while keeping the arms along the body and the non-dominant in suspension with the knee flexed. The task was executed in three experimental sessions (description in detail below) and consisted of 10 trials in each single session. Furthermore, there was a between trial rest period of 30 seconds, in which

subjects could stand on both legs. The subject's initial upright position was calculated by using the first 3 seconds of each trial. This value was used as reference to trace the quantity of leg displacements throughout the rest of the trial.

Inspired by Dejnabadi et al. (2006), subjects' movements were recorded using a custom-made three-dimensional accelerometer (ADXL345) put on the dominant lower limb and connected to a microcontroller panel. In the range of low frequency movements like normal gait and single-leg stance task, the joint angles can be measured precisely using an accelerometer (Chang et al., 2012; Neville et al., 2015; Perez-Cruzado et al., 2014). This system allows to obtain a proper and high-resolution detection of subjects' movement sways (with a precision of 0.04 degrees). Furthermore, the system is characterized by high flexibility, being made by a friendly device easy to apply, to remove and to transport. These features make the system a potential device to easily analyse balance outside the laboratory, such as in the clinical setting for the study of patients with postural deficit.

Data were stored for offline analysis with Matlab (Matlab 2014, MathWorks). A PC monitor was located in front of the participants at a distance of 100 cm during the task. The monitor was used to give the instructions to the participants and as fixation frame during the trials. Furthermore, participants were barefoot during the task, to avoid any confounding effect on the balance task due to the type of shoe.

Procedure

Before starting the experimental procedure, participants did a training of 2 small trials (15 seconds each) to familiarize with the task. During the experiment, the balance task was performed three times: T0, T1, T2 (Figure 8A). After familiarizing with the task, participants performance T0, which represented the baseline and allowed to get a measure of performance before any manipulation. Then, the placebo treatment was applied and consisted of the application of transcutaneous electrical nerve stimulation (TENS) on one of the muscles of the dominant leg implicate in the balance task (grastrocnemius muscle) for 3 minutes while participants were seated. The TENS's intensity was regulated until subjects perceived a slight sensation on the skin without muscle contraction. The frequency of TENS was set at 10 Hz and was entirely inert in producing any active

modification of balance performance. Along with the inert treatment, subjects of the experimental group were told that TENS had the effect of increasing the recruitment of muscle fibers and so improving balance control. Conversely, participants of the control groups underwent the same inert stimulation, but with different verbal information about TENS. Precisely, they were clearly told that they belong to a control group in which TENS was to be applied with an inactive mode. Right after the first TENS application, participants underwent the T1. To investigate the potential strengthening of the placebo effect, the same TENS application was repeated as before. The experimenter reminded subjects about the positive (experimental group) benefit or inactive mode (control group) of the treatment on the balance task. Subjects finished the experiment by performing the T2.

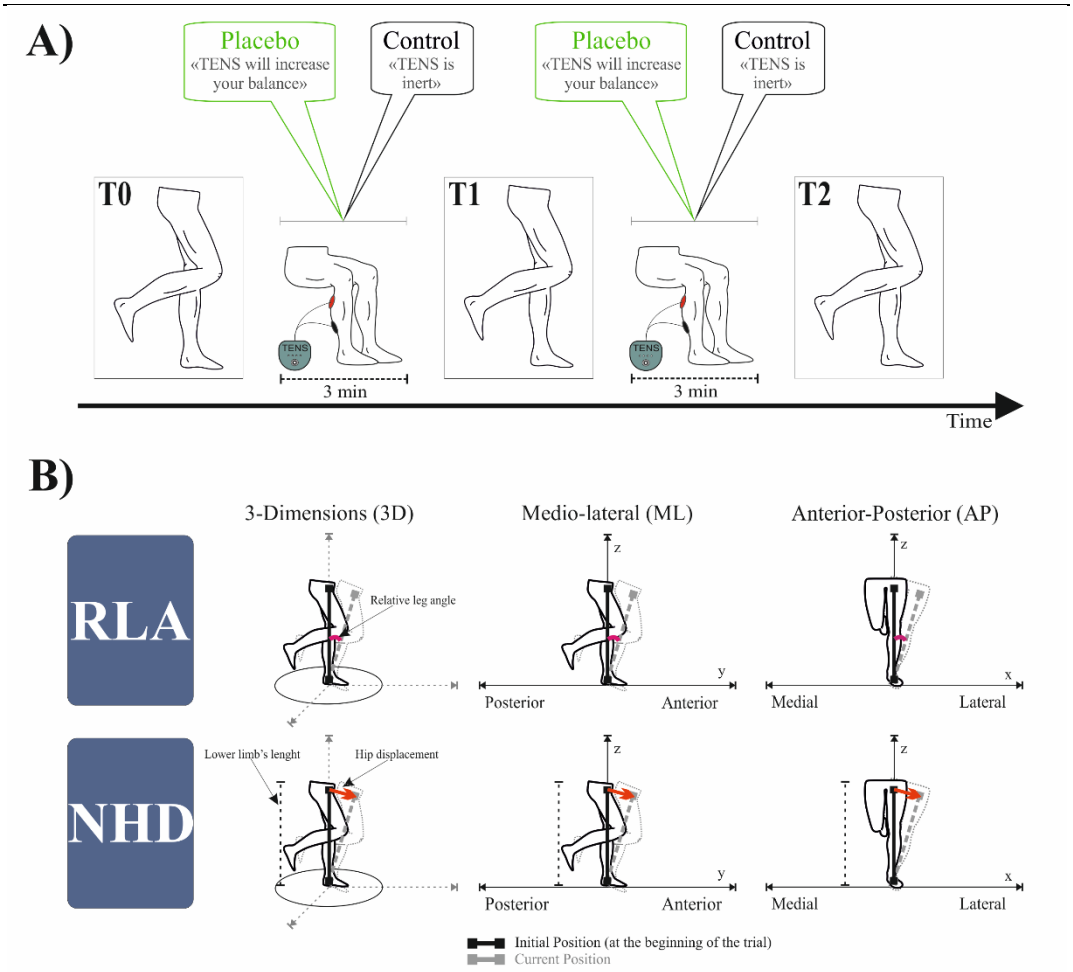

Figure 8. Representation of the experimental protocol and the defined indexes. (A) The procedure consisted of three sessions (T0, T1 and T2). In each session, participants performed a single-leg balance task by standing as steadily as possible with the dominant

leg for 30 seconds while the non-dominant leg was kept in suspension. Subjects repeat the described task 10 times for each session. Before T1 and T2, transcutaneous electrical nerve stimulation (TENS) treatment was applied on the dominant leg while subjects were seated for 3 minutes. Different verbal information about the effects of TENS was given to the placebo and control group. (B) Representation of the definition and computation of the indexes RLA and NHD. RLA (relative leg angle) was defined as the angular deviation (in degrees) of the dominant leg (gray-dashed lines on the leg's figures) with respect to its initial upright position (black lines on the leg's figures) measured in the calibration. Maximum values of RLA were taken in the three-dimensional space (RLA-3D), in the medial-lateral direction (RLA-ML) and the anterior-posterior direction (RLA-AP). NHD (normalized hip displacement) consisted of the displacement of the subject's hip (gray-dashed lines on the leg's figures) with respect to the initial position (black lines on the leg's figures) normalized to the length of the subject's lower limb. Maximum values of NHD were taken in the three-dimensional space (NHD-3D), in the medial-lateral direction (NHD-ML) and the anterior-posterior direction (NHD-AP). The figure illustrates the movement in one direction as example, real sway displacements might have occurred in both direction and dimensions.

Measures of Performance

Behavioural parameters

Leg movements and spatial position recorded through the accelerometer were considered as proxy of balance control. Specifically, two main parameters were derived: relative leg angle (RLA) and normalized hip displacement (NHD).

RLA is defined as the angular deviation (in degrees) of the dominant leg with respect to its initial upright position measured at the beginning of each trial (Figure 8B). To characterise the subject's movements in a fine-tuned way, different indexes were extracted from RLA. Particularly, we measured the maximal postural sway defined as the maximum RLA obtained in the three-dimensional space (RLA-3D). High values of RLA-3D are indicative of large postural sways. We also derived the total amount of movement variability defined as the standard deviation of RLA in the three-dimensional space ($RLA\text{-}3D_{std}$). Higher values of $RLA\text{-}3D_{std}$ are indicative of higher postural variability. Finally, we derived the body sways in specific directional planes, such as the maximum RLA in the medial-lateral direction (RLA-ML) and the maximum RLA in the anterior-posterior direction (RLA-AP).

The NHD parameter consisted of the displacement of the subject's hip (in cm) with respect to the initial position, normalized to the length of the subject's lower limb, according to the formula:

$$NHD = \frac{HD}{leg\ length} \times 100$$

Similar to RLA, several indices were extracted to deeply analyse subject's movements. NHD-3D is defined as the maximum NHD obtained in the three-dimensional space and NHD-$3D_{std}$ is defined as the NHD standard deviation in the three-dimensional space. In addition, NHD-ML and NHD-AP were also measured to better characterize movement directions in the medial-lateral and anterior-posterior directions, respectively (Figure 8B).

For all the indexes, the mean of the 10 trials was calculated in each session. Higher values in these indexes indicate worse balance control.

Subjective parameters

Subjective variables were also assessed throughout the procedure. Particularly, we measured the subjective perception of stability by asking participants to judge how stable they have felt on a 10 cm visual analogue scale (VAS) ranging from 0 (very unstable) to 10 (very stable) after completed the balance task at the end of each session. Furthermore, we assessed the subjective sense of extent by asking participants to complete the Borg scale, ranging from 0 (rest) to 10 (maximal effort) (Borg et al., 1982) after each trial in each session, thus monitoring cautiously the sense of effort during the whole experimental procedure. To measure the expectation about the effects of TENS, participants were asked to judge whether they expected an improvement or worsening of performance on a number rating scale (NRS) ranging from -3 (much worse than at baseline) to +3 (much better than at baseline), with 0 (the same as at baseline) soon after each TENS application (before task execution). Finally, we measured the perception of treatment efficacy by asking participants to judge whether TENS was effective or not in enhancing stability on a 10 cm VAS ranging from 0 (not effective at all) to 10 (extremely effective) after the conclusion of the balance task at T1 and T2.

Statistical Analysis

Behavioural (RLA-indexes and NHD-indexes) and subjective parameters (perception of balance, sense of effort, expectation, perception of treatment efficacy) were analysed using SPSS Statistics 21 software (IBM SPSS Statistics 21, SPSS Inc., Chicago, IL). Normality of data distribution was checked with the Shapiro-Wilk test. All the variables analysed violated the normal distribution of data ($p < 0.050$), for this reason non-parametric analyses were performed. Mann-Whitney U test was used to compare the two groups (Placebo vs. Control) in each session separately (T0, T1, T2). Moreover, the test of Friedman was applied to analyse the factor Session (T0, T1, T2) within each group separately. Post-hoc comparisons were carried out with Wilcoxon signed rank test, just in the case of significant factor.

The effect size of all the results were analysed with the Cohen's *d* statistic (Cohen, 1988). Bonferroni correction for multiple comparisons was used when needed and the level of significance was set at $p \leq 0.05$. Data are represented as median values.

Results

As aforementioned, height and foot size were matched between groups to exclude any bias regarding these variables on the balance motor task. The two groups did not statistically differ for height ($p = 0.810$) or foot size ($p = 0.800$).

Relative Leg Angle (RLA)

The Mann-Whitney test on RLA-3D showed that the placebo group had a lower range of postural sway (Mdn = 2.23) than the control group (Mdn = 2.78) at T2 ($U = 60.0$, $p = 0.029$, $d = 0.87$). However, no difference between the two groups was found at T0 and T1 (for both comparisons, $p > 0.345$). The within-subject analysis did not disclose any significant difference across sessions for the placebo ($p = 0.15$) and control group ($p = 0.62$) (Figure 9A).

Concerning $RLA\text{-}3D_{std}$, no difference was found between the placebo and the control group ($p > 0.064$), nor in the within-subject analysis in both groups ($p > 0.28$) (Figure 9B).

With regards to RLA-ML, the placebo group displayed significant lower values (Mdn = 1.22) than the control group (Mdn = 1.36) at T2 (U = 64.0, p = 0.044, d = 0.77). Nonetheless, both groups were comparable at T0 and T1 (for both comparisons, p > 0.12). The Friedman test did not display any significant difference across sessions in the placebo (p = 0.11) or in the control group (p = 0.88) (Figure 9C).

Analysis of RLA-AP revealed no differences between the placebo and control group (p > 0.056). On the contrary, the placebo group displayed a significant effect of Session (χ^2 = 6.4, p = 0.041). Post-hoc analyses showed that participants of the placebo group obtained a significant higher balance control at T2 (Mdn = 1.45) than at T1 (Mdn = 1.65) (Z = 2.67, p = 0.032, d = 1.09) (Figure 9D).

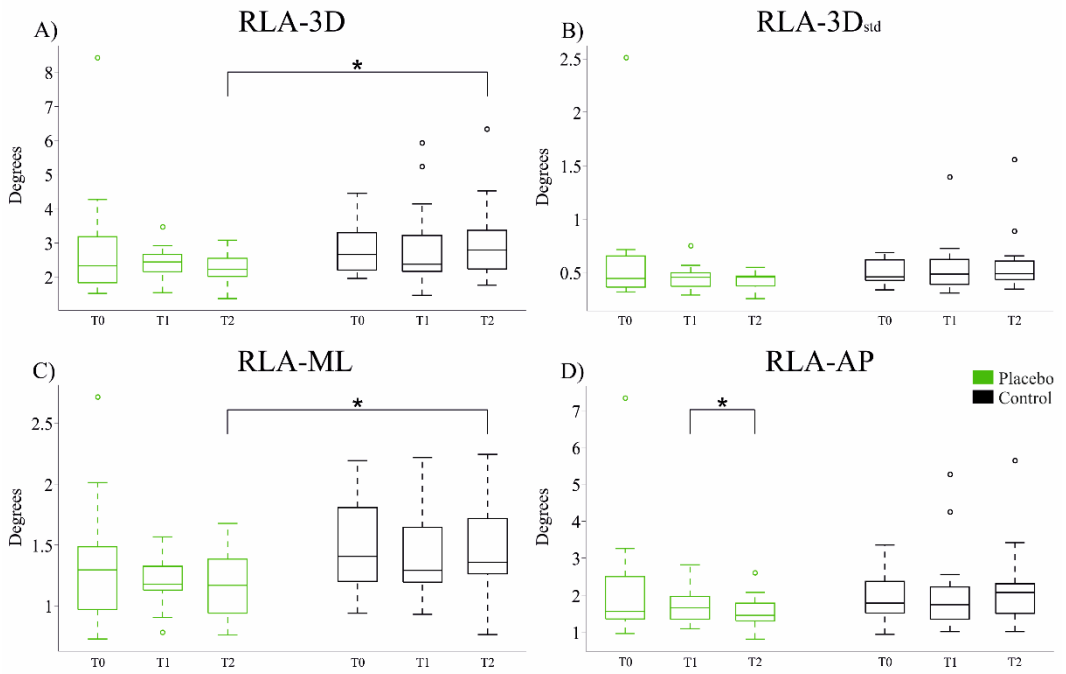

Figure 9. Box plots of the behavioural data for the relative leg angle (RLA). **(**A) RLA-3D was lower in the placebo group compared to the control group at T2. (B) Similarly, RLA-$3D_{std}$ was lower in the placebo group compared to the control group at T2. (C) RLA in the medial-lateral direction (RLA-ML) was lower in the placebo group than the control group at T2. (D) RLA in the anterior-posterior direction (RLA-AP) was lower at T2 compared to T1 only in the placebo group. *Significant values (p < 0.05); ° outliers (values that were located outside 1.5 times the interquartile range above the upper quartile and below the lower quartile); Horizontal lines represent median values.

Normalized Hip Displacement (NHD)

The Mann-Whitney test on NHD-3D revealed lower hip's displacement in the placebo group (Mdn = 2.68) than in the control group (Mdn = 3.69) at T2 (U = 56.0, p = 0.019, d = 0.92) while the two groups did not differ at T0 and T1 (for both comparisons, $p > 0.45$). The Friedman test revealed a significant effect of Session for the placebo ($\chi^2 = 6.13$, $p = 0.047$) and not for the control ($p = 0.760$) group. Post-hoc tests showed that participants obtained a significant improvement of balance at T2 (Mdn = 2.68) compared to T1 (Mdn = 2.94) in the placebo group (Z = 2.10, p = 0.036, d = 0.38) (Figure 10A).

Regarding NHD-$3D_{std}$, the placebo group displayed significantly lower variability (Mdn = 0.72) than the control group (Mdn = 0.81) at T2 (U = 63.5, p = 0.04, d = 0.79). However, no difference between the two groups was found at T0 and T1 ($p > 0.48$). The within-subjects analysis did not show any significant effect of Session within the two groups ($p > 0.28$) (Figure 10B).

With regards to NHD-ML, lower values were found in the placebo group (Mdn = 1.75) compared to the control group (Mdn = 2.01) at T2 (U = 63.5, p = 0.042, d = 0.79). However, the two groups were comparable at T0 and T1 (for both comparisons, $p > 0.18$). The Friedman test did not show significant effects in the placebo ($p = 0.81$) and control ($p = 0.42$) groups (Figure 10C).

The analysis of the NHD-AP showed no differences between the placebo and the control ($p > 0.056$). Conversely, the Friedman test displayed a significant effect of Session only in the placebo group ($\chi^2 = 8.4$, $p = 0.015$) and not in the control group ($p = 0.76$). Post-hoc analysis showed that participants obtained a significant improvement of balance at T2 (Mdn = 2.25) compared to T1 (Mdn = 2.51) in the placebo group (Z = 2.64, p = 0.032, d = 0.47) (Figure 10D).

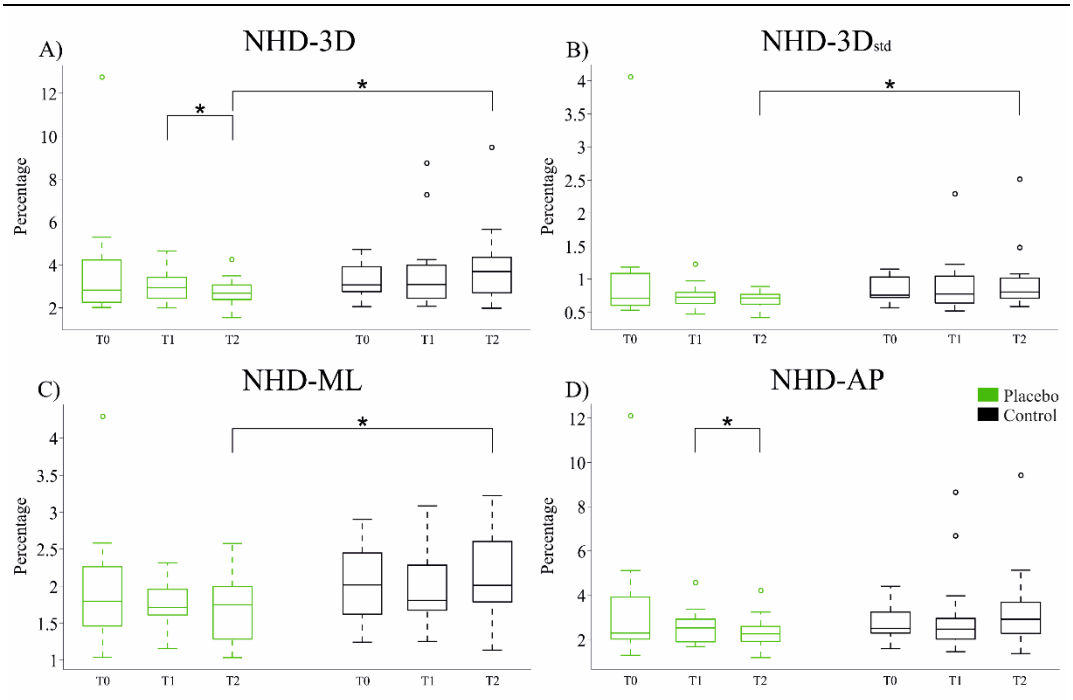

Figure 10. Box plots of the behavioural data for the normalized hip displacement (NHD). (A) In the placebo group, NHD-3D was lower at T2 compared to T1 and compared to the control group (B) NHD-$3D_{std}$ was lower in the placebo than in the control group at T2. (C) In the same way, NHD-ML was lower in the placebo than in the control group at T2. (D) In the placebo group, NHD-AP was lower at T2 compared to T1. *Significant values ($p < 0.05$); °outliers (values falling between 1.5 and 3 times above or below the interquartile range); Horizontal lines represent median values.

Subjective parameters

With regards to the perception of stability, higher perception of stability was found in the placebo group (Mdn = 8.6) compared to the control group (Mdn = 7.3) at T2 (U = 60.0, $p = 0.029$, d = 0.87). However, the two groups did not differ at T0 and T1 ($p > 0.16$). The within-subjects analysis revealed a significant effect of Session for the placebo group ($\chi^2 = 10.4$, $p = 0.005$) and not for the control group ($p = 0.94$). Post-hoc comparisons showed that subjects perceived themselves as more stable at T2 (Mdn = 8.6) compared to T0 (Mdn = 7.5) in the placebo group (Z = 2.75, $p = 0.018$, d = 1.15) (Figure 11A).

The analysis of the sense of effort (Borg) did not reveal significant differences between groups in any session ($p > 0.96$). In a similar way, the Friedman test display no significant difference within groups ($p > 0.88$).

Concerning, the analysis of expectation, participants of the placebo group obtained higher expectation of improvement (Mdn = 1.0) than participants of the control group (Mdn = 0.0) soon after the first TENS application (U =26.0, $p < 0.001$, d = 1.96). Similarly, subjects expected more improvement in the placebo group (Mdn = 1.0) than in the control group (Mdn = 0.0) after the second TENS application (U =42.0, $p = 0.001$, d = 1.42) (Figure 11B).

Regarding TENS efficacy, subjects of the placebo group perceived the TENS as more effective (Mdn = 6.7) than those of the control group (Mdn = 1.4) when they were asked at the end of T2 (U = 51.2, $p = 0.01$, d = 1.03). However, no difference in TENS efficacy scores was found between groups at the end of T1 ($p = 0.08$). The Wilcoxon test showed significant differences in the placebo group (Z = 2.41, $p = 0.016$, d = 0.98), showing higher TENS efficacy scores after T2 (Mdn = 6.7) than T1 (Mdn = 5.0). No differences were found for the control group ($p = 0.58$). This result suggests that the placebo manipulation worked successfully due to the increase of the perception of TENS efficacy during the experiment in the placebo group (Figure 11C).

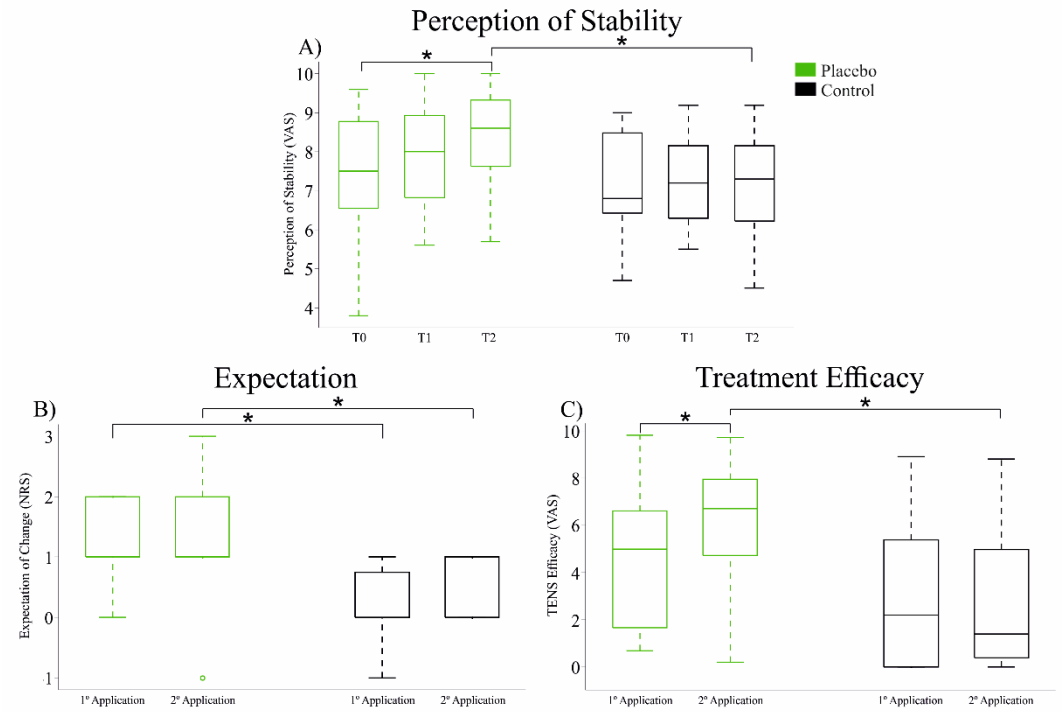

Figure 11. Box plots of the subjective data. (A) Perception of stability in the placebo group at T2 was higher compared to T0 and compared to the control group. Moreover, subjects in the placebo group perceived themselves as more stable at T2 than at T0. (B) Subjects of the placebo group expected to be more stable compared to subjects of the control group after both TENS applications. (C) Subjects of the placebo group perceived more effect of TENS after the second than the first application. Moreover, after the second application subjects of the placebo group perceived more effects of TENS than the control group. *Significant values ($p < 0.05$); °outliers (values that were located outside 1.5 times the interquartile range above the upper quartile and below the lower quartile); Horizontal lines represent median values.

Discussion

In the present study we aimed at investigating whether the placebo effect could be induced in a monopodalic balance task. Our results show for the first time that balance control can be enhanced with a placebo procedure consisting of verbally-induced positive expectations about the efficacy of a treatment.

Placebo effect enhances balance control

Our findings indicate that participants of the placebo group had a general decrease of body sways compared to the control group, suggesting a better postural control. The enhancement of balance was found consistently in the indexes derived from

the relative leg angle and the normalized hip displacement. Specifically, the values of RLA and NHD of the placebo group were lower than those of the control group specifically at T2, indicating that the placebo treatment needs to be applied at least twice to induce significant improvements on balance. A significant difference between groups was found overall in the capacity to maintain balance in the three-dimensional space (i.e., RLA-3D and NHD-3D). The difference between groups was preserved for both indexes in the ML (but not in the AP) direction, with a smaller amount of body sways in the placebo group than in the control group at T2, while in the AP direction a within-group difference from T1 to T2 was found in the placebo group.

These results are in accordance with the idea that body sways in the ML and AP directions represent different components of postural stability and also demand different cortical activation to be well controlled (Mochizuki et al., 2006; Slobounov et al., 2008). Generally, sways in the AP direction are greater in magnitude than sways in the ML direction (Bustamante Valles et al., 2006; Gage et al., 2004; Mochizuki et al., 2006). This pattern appears to be present also in our study and it could explain why a within-group reduction from T1 to T2 could more easily emerge in the AP direction. Of note, displacements in the ML direction have been usually related to higher postural instability, with consequent risk of falls, and have been associated to task difficulty both in healthy individuals and in pathological populations (Bustamante Valles et al., 2006; Gatev et al., 1996; Maki et al., 1994; Mochizuki et al., 2006). Therefore, finding less ML sways in the group of participants who underwent a placebo procedure compared to controls hints at a potential translational impact of this approach to persons at risk of falls, like the elderly or cerebellar patients. The mechanisms underneath these outcomes have not been investigated in our study, albeit it is reasonable to speculate that the placebo-induced improvement of balance in the ML direction could be associated to the involvement of cortical sensorimotor regions. Particularly, a previous study demonstrate that the control of the ML balance sway is related to the activation of cortical sensorimotor regions (Slobounov et al., 2008).

Regarding the subjective variables, we found that perception of stability was in accordance with the behavioural results, in that participants of the placebo group

perceived higher stability across sessions and compared to the control group. This suggests that the placebo procedure could modify both behavioural and subjective parameters (Rossettini et al., 2018; Schwarz & Buchel, 2015). Expectations scores of the placebo group were significantly higher compared to the control group after the two applications of the TENS treatment, indicating that the verbal information about TENS was effective in inducing positive expectations of better performance. Perception of TENS efficacy was also higher in the placebo group, suggesting that participants of the placebo group believed in the efficacy of the TENS.

The application of the inert TENS treatment to both groups permits to dismiss any influence on balance control because of the simple leg stimulation. The lack of improvement in the control group may be attributed to the difficulty of the monopodalic task, which have a tendency to produce higher postural instability than bipedal tasks (Bisson et al., 2010; Vuillerme et al., 2001). Owing to the absence of difference between groups in the BORG scale, the subjective sense of effort does not seem to have influenced the performance at the task.

Overall, our findings indicate that balance control and perception of stability can be improved by a placebo procedure.

Balance control and brain activity

Postural control is a complex motor skill based on the interaction between different sensorimotor systems (Horak, 2006) and on the activity of cortical and subcortical brain regions (Jacobs & Horak, 2007a; Mierau et al., 2017; Wittenberg et al., 2017). Typically, postural control has been thought to be mainly controlled by sub-cortical brain regions of the brainstem (Magnus, 1926). Additional recent studies support a different conception that hint at the involvement of the cerebral cortex (see Jacobs & Horak, 2007a and Wittenberg et al., 2017). Particularly, two main circuits seem to be relevant for postural control: a cortical-cerebellar loop and a cortical-brainstem loop involving the basal ganglia (Jacobs et al., 2005; Jacobs & Horak, 2007a; Takakusaki, 2017; Timmann & Horak, 1997).

Studies with electroencephalography and transcranial magnetic stimulation indicate that a set of cortical regions may be involved in diverse aspects of postural control, like the primary motor cortex, the supplementary motor area, the cingulate cortex,

the parietal, temporal and insular cortex (Dimitrov et al., 1996; Duckrow et al., 1999; de Waele et al., 2001; Jacobs & Horak, 2007a; Jacobs et al., 2008; Quant et al., 2004; Taube et al., 2006). These regions concur in the integration of sensory input (mainly vestibular and somatosensory) needed for optimal postural control, as well as in the pre-selection and optimization of postural responses needed to anticipatorily control an eventual loss of balance (Ackermann et al., 1991; de Waele et al., 2001; Ghafouri et al., 2004; Horak et al., 1996; Jacobs & Horak, 2007b; Zettel et al., 2005). Studies on the cortical-muscular coherence have found a relation between cortico-muscular activity and postural control (Jacobs et al., 2015; Watanabe et al., 2018). Moreover, electrophysiological studies have demonstrated the critical role of motor cortical areas and of the corticospinal system in balance control (Soto et al., 2006; Taube et al., 2006; Tokuno et al., 2009). Of note, postural responses involve the activation of muscle synergies throughout the entire body (Bernikera et al., 2009; Horak & Macpherson, 1996; Jacobs & Horak, 2007a; Torre-Oviedos & Ting, 2010; Wojtara et al., 2014) and the motor cortex plays a relevant role in activating these muscle synergies (Holdefer et al. 2002; Leo et al., 2016; Rana et al., 2015). Interestingly, a previous study with transcranial magnetic stimulation revealed that a placebo procedure could modulate the activity of the primary motor cortex, enhancing the excitability of the corticospinal system (Fiorio et al., 2014). Therefore, founded on this evidence we speculate that our placebo intervention could have optimized the corticospinal control of muscle synergies, thus resulting in better balance performance.

Brain regions involved in anticipatory postural control could also play a role. Regarding this, it was shown that anticipated postural perturbations are related to changes in the readiness potential (Jacobs et al., 2008; Jacobs & Horak, 2007a). The readiness potential is an electrophysiological sign of cortical excitability registered before voluntary movement onset and seems to be generated in the sensory-motor cortex and in the supplementary motor area (Deecke, 1996; Shibasaki & Hallett, 2006). An earlier study discovered that the readiness potential could be modulated by placebo procedures and this modulation was related to the placebo-induced reduction of fatigue (Piedimonte et al., 2015). Therefore, it could also be suggested that the placebo intervention in our study influenced the brain regions involved in

the anticipatory control of posture in order to better prevent a possible loss of balance.

Our task was not based on the use of perturbations to measure subjects' postural control (the so-called dynamic balance), but on a continuous intentional control of balance in a monopoladic stance. This type of task most likely involves voluntary control and cortical centres (Hülsdünker et al, 2015; Obata et al., 2009; Slobounov et al., 2009; Tokuno et al., 2009). Therefore, founded on the abovementioned studies, we would suggest that cortical regions involved in voluntary postural control could play an important function in the placebo-induced enhancement of balance. This hypothesis should be proved in future studies by applying neurophysiological techniques.

The placebo effect on motor learning

According to our previous study, we might add a new aspect of motor performance that can be enhanced by a placebo procedure. Nonetheless, there are still many different aspects of motor performance that remain without an answer to whether a placebo procedure may modulate them. In this second behavioral study, we focused on one crucial motor function that is present in the activities of our daily life, the so-called motor skill learning.

Motor skill learning refers to the enhancement of specific movements after several repetitions to achieve a motor coherent behaviour (Dayan & Cohen, 2011). Motor skill learning is present in many daily activities and entails the acquisition of a new motor skill in a better, faster and accurately manner (Diedrichsen & Kornysheva, 2015). Several studies have demonstrated that the process of learning a new motor skill is divided in three stages. A first early stage, where there is a significant improvement of learning within the first session of training. A second stage, where the amount of improvement is small and can be observed between sessions separated over days or weeks. Finally, an intermediate stage, the so-called consolidation period, where the repeated movements become more stable and it occurs between sessions separated by more than 6 hours (Doyon et al., 2003; Karni et al., 1998). The learning of a new motor skill is assessed by the speed-up of reaction time and the reduction of number of error (Willingham, 1998).

The two tasks that have been most frequently adopted to experimentally investigate motor skill learning are motor adaptation and motor sequence learning. Motor adaptation refers to the modification of a well-learned skill due to external changes (Wolpert et al., 2011; Diedrichsen & Kornysheva, 2015). Motor sequence learning refers to the explicit or implicit learning of a sequence of movements, typically performed with the fingers (Hirano 2018; Korman et al., 2003; Robertson et al., 2004; Willingham, 2001).

In the present study, we planned to investigate whether the placebo effect can have an impact on motor skill learning. More precisely, we decided to focus on the learning of a motor sequence, which is a very important function in many daily life activities. One of the most used paradigms to investigate the learning of a motor sequence is the serial reaction time task (SRTT) (Nissen and Bullemer, 1987). The

SRTT consists of a visual stimulus that emerges in one of four different positions on a PC screen. Subjects should press with one of four fingers the button of a key-box corresponding to the position of the visual stimulus. Specifically, the first position of the visual stimulus on the screen corresponds to a key press with the index finger, the second position on the screen corresponds to a key press with the middle finger and so on to the little finger. A deterministic sequence of 10-12 stimuli positions is typically used and hidden between two blocks of random positions. Hence, the SRTT allows to study the implicit learning of a motor sequence in which subjects are unaware about the existence of a deterministic pattern (Schwarb & Schumacher, 2012). The use of the SRTT allows to measure the learning of a motor sequence as well as the consolidation of that motor sequence. Furthermore, it can also distinguish two components of sequence motor learning: the general motor performance (the improvement of response time in random stimuli due to an aspecific effect of learning); and sequence learning or skill learning (the improvement due to the difference between random and sequence blocks) (Perez et al., 2007; Robertson, 2007). With regards to memory consolidation, the SRTT allows to evaluate the off-line learning of these two components (general motor performance and skill learning) across time (King et al., 2013).

Many studies have investigated the neural correlates of motor sequence learning in young adults (Ashe et al., 2006; Doyon et al., 2003; Doyon & Benali, 2005; Gheysen et al., 2010; King et al., 2013). It has been found that the activation of different cortical and subcortical brain areas contributes to the process of motor skill learning, depending on the stage of learning (Ashe et al., 2006; Dayan & Cohen, 2011; Doyon & Benali, 2005; King et al., 2013), suggesting a hierarchical organization (Diedrichsen & Kornysheva, 2015). Moreover, several areas such the primary motor cortex (M1), the pre-supplementary and the supplementary motor area (SMA), the premotor cortex (PM), the striatum (basal ganglia) and the hippocampus seem to be active during the fast learning phase. Instead, during the consolidation phase, the motor sequence learning relies on the activity of the cortico-striatal circuitry (Doyon et al., 2003; Doyon et al., 2009; Gheysen et al., 2010; Hardwick et al., 2013; Kim & Shim, 2014; King et al., 2013; Rauch et al.,

1997). Among all these brain regions, the activity of M1 seems to be important because it sends the motor command of the learned movement and it produces faster and more precise movements due to the control of muscle synergies (Harwick et al., 2013; Krakauer & Mazzoni, 2011; Penhune & Steele, 2012). Hence, we could hypothesize that if the placebo effect in the motor domain can enhance the excitability of M1, as previously demonstrated (Fiorio et al., 2014), then we should be able to induce an improvement of motor skill learning.

Motor skill learning, however, does not rely only on motor functions but also on cognitive functions (Janacsek, & Nemeth, 2012; Kaufman et al., 2010). Additionally, it has been proposed that motor sequence learning is relevant in rehabilitation, training or educational programs (Howard et al., 2004; Janacsek & Nemeth, 2012; Nemeth et al., 2010). Hence, it is reasonable to think that a placebo procedure in this domain may have an effect because it improves the deployment of cognitive resources.

In this study, we address how the placebo effect, consisting of verbal suggestion, can modulate the learning of a sequence of fingers movements with the serial reaction time task. To tackle the specific contribution of motor or cognitive functions in the placebo effect on sequence learning, we applied a placebo procedure directed either to the improvement of motor functions or of cognitive functions.

Method

Participants

Eighty-nine healthy right-handed participants were recruited (43 females; mean ± SD; 21.0 ± 2.31 years) from the student population of the University of Verona. Participants were divided in three different groups: 30 subjects (15 females; mean age: 20.6 ± 2.12 years) were enrolled in the Placebo-tDCS group; 30 subjects (15 females; mean age: 20.7 ± 2.15 years) were enrolled in the Placebo-TENS group; and 29 subjects (13 females; mean age: 21.7 ± 2.56 years) were enrolled in the Control group.

Participants were informed about the experiment procedure, but unaware of the placebo nature of the study. Participants signed a written informed consent at the

beginning of the experiment and were debriefed about the placebo nature of the study only after having completed all the experimental sessions.

Serial reaction time task

A modified version of the SRTT (Nissen & Bullemer, 1987) was created with E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA). A visual cue was presented on the PC monitor (34 x 27cm) and consisted of a filled blue rectangle (Figure 12). The cue showed up in one of four possible positions distributed equally in a horizontal array. Each cue position corresponded to one of four buttons of a response pad (Chronos device, Psychology Software Tools, Pittsburgh, PA). Subjects positioned four fingers of the right hand on the four buttons of the response pad, with the index finger on the first left button and the little finger on the last right button. The distance between the monitor and the subjects' eyes was 70 cm.

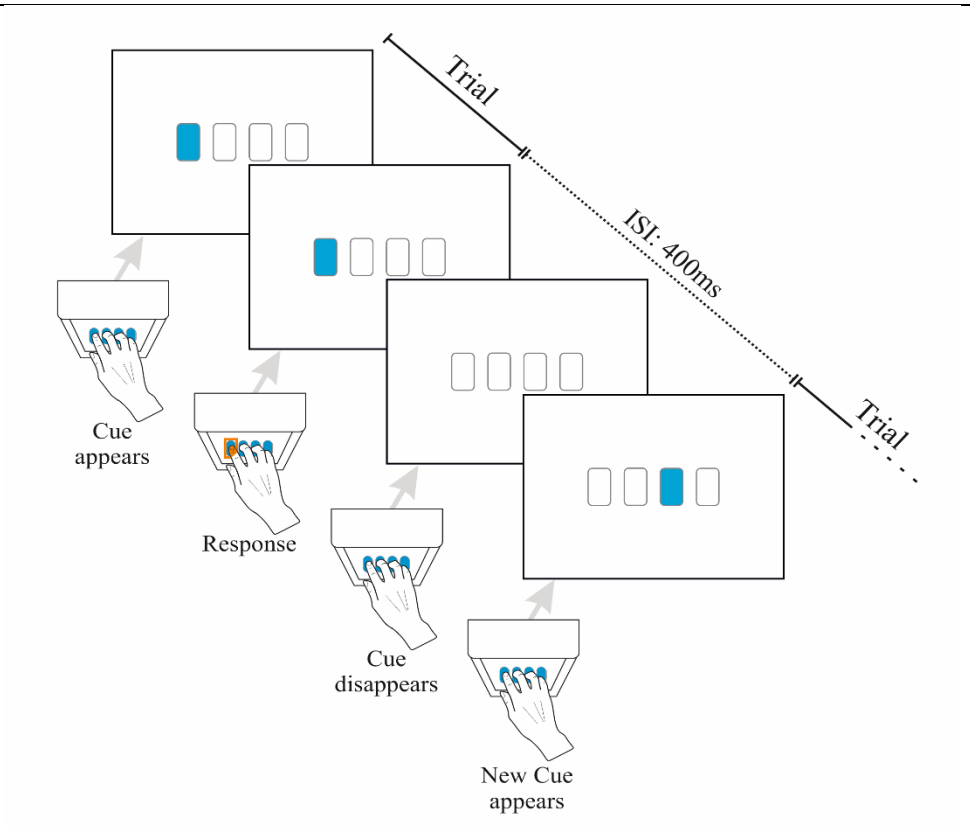

Figure 12. Representation of a trial of the serial reaction time task. After the appearance of a visual cue on the PC monitor, subjects had to respond by clicking the appropriate response button on a response box. After the correct button press, the visual cue disappeared. After 400ms of inter-stimulus interval (ISI), another trial started with another

visual cue. The task was similar for sequence and random trials; the only difference was that the position of the visual cue could follow a deterministic sequence or a random succession.

Subjects were told to respond as quickly and accurately as possible by pressing the button on the pad that corresponded to the position of the visual cue. When the subjects' response was correct, the visual cue disappeared. After that, another visual cue showed up with an inter-stimulus interval of 400ms (Figure 12). Conversely, when subjects' response was incorrect the visual cue remained on the screen until a correct answer was given. Unbeknown to the subject, there was a special session in which the position of the visual cue followed a repetitive 12-items sequence. This deterministic sequence was the same for all subjects (2-3-1-4-3-2-4-1-3-4-2-1). To maintain the sequence hidden from explicit awareness, the block entailing the sequence was preceded and followed by blocks with the random positions. Since the position of the cue corresponds to the movements of the fingers, the assumption is that if the hidden sequence is implicitly learned we should observe faster reaction times in this case compared to the random positions.

The SRTT was performed in three sessions (baseline, learning, final). The baseline session consisted of 15 repetitions of the deterministic sequence (180 trials), the learning session contained 25 repetitions of the deterministic sequence (300 trials) and the final session was similar to the baseline. Fifty random trials were placed before and after each group of sequence in each session (Figure 13). Each set of 50 random trials was unique for each session, but it was identical across subjects and groups. To create the random trials, attention was paid to avoid repetitions of the same visual cue position (e.g. 2-2) and to avoid "salient" sequences (like 1-2-3-4) (Brown & Robertson, 2007; Önal-Hartmann et al., 2012). Moreover, the cue appeared the same number of times in each of the four positions.

Procedure

At the very beginning of the experimental procedure, all subjects performed a small training to familiarize with the SRTT. In each group, the experiments consisted of three sessions: baseline, learning and final (as described above). The baseline and the final sessions were the same.

Once the participants have completed the baseline session, we introduced a placebo procedure. One group of participants underwent a placebo procedure directed to the motor component of the task. More precisely, this group (called Placebo-TENS group), received a placebo treatment consisting in the application of transcutaneous electrical nerve stimulation (TENS) on the muscles involved in the SRTT, specifically the first dorsal interosseous and the abductor digiti minimi for 5 minutes. The intensity of TENS was adjusted until subjects felt a slight sensation on the skin without muscle contraction. The frequency of TENS was set at 10 Hz and did not induce any active modification of performance *per se*. Along with TENS, subjects received a verbal suggestion of its positive effect in enhancing the efficiency of the hand muscles involved in the task.

Another group of participants underwent a placebo procedure directed to the cognitive component of the task. More precisely, this group (called Placebo-tDCS group), received a placebo treatment consisting in the application of sham transcranial direct current stimulation (tDCS) on the frontal area for 5 minutes. The anode and cathode electrodes were placed above the left and right eyebrows, respectively. The electrodes (5 x 5 cm) were inserted into a sponge soaked in saline solution (0.09% Na). The sham tDCS was applied with 30 seconds of active stimulation at the beginning and at the end of the application period (with a ramp up/down of 10 seconds) and was automatically turned off during the rest of the application. tDCS intensity was set at 1mA. This sham protocol has been demonstrated to induce the sensation of being stimulated without a real modulation of the target area (Gandiga et al., 2006; Nitsche et al., 2008). Together with tDCS, subjects received a positive verbal suggestion of enhancement of concentration and attention that are required to perform well in the task. After the placebo treatment was applied, participants performed the learning session.

To investigate the potential strengthening of the placebo effect, both TENS and tDCS placebo treatments were applied again between the learning and the final session. Then, participants performed the final session. Participants of the control group (natural history) underwent the same three sessions, but without any treatment. After each session, subjects were told to wait for 5 minutes before starting the next session (Figure 13).

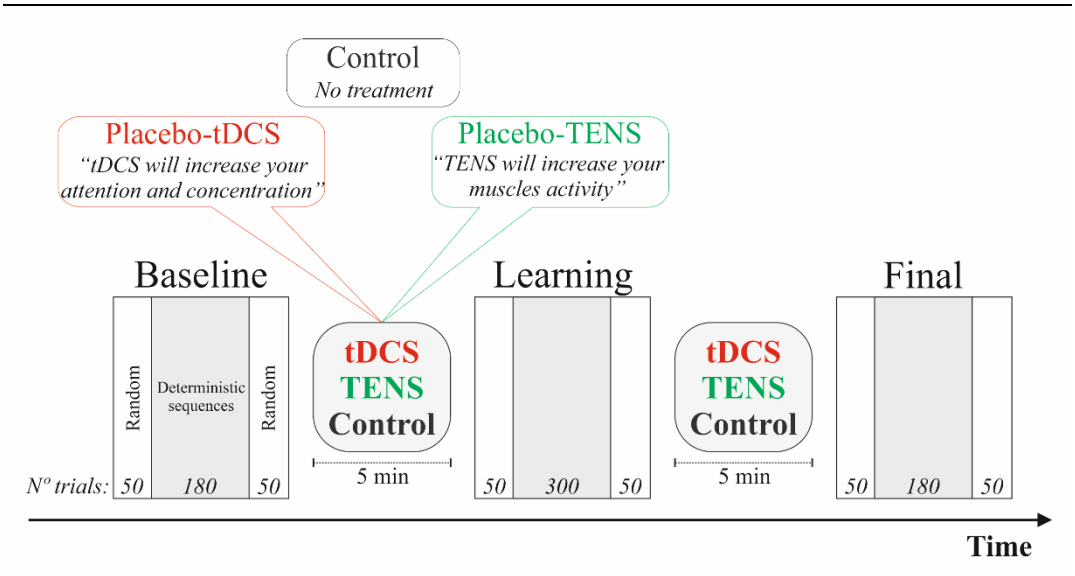

Figure 13. Schematic representation of experimental protocol. The procedure consisted of three sessions (baseline, learning and final). In each session, participants performed the SRTT as quickly and accurately as possible. After the baseline session was completed, both experimental groups underwent a placebo procedure. The Placebo-TENS group received a placebo treatment consisting in the application of transcutaneous electrical nerve stimulation (TENS) on the muscles involved in the SRTT (right hand), while the Placebo-tDCS group received a placebo treatment consisting in the application of sham transcranial direct current stimulation (tDCS) on the frontal area. Along with TENS and tDCS application, participants received different verbal suggestion, according to each experimental group. Before the final session, the placebo treatment was applied for a second time in both experimental groups with the same verbal information. Participants finished the SRTT by performing the final session. Participants of the control group (natural history), performed the same three sessions, without any treatment.

Motor and Visual Recognition tasks

The ability of all subjects to remember and recall the sequence was evaluated at the end of the overall experiment. Two open question were made "*Do you have anything to report regarding the task?*" and then "*Did you notice anything special about the task?*".

Moreover, to control whether participants were aware of the sequence, two recognition tasks (one motor and one visual) programmed with E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA) were included at the end of the experiment. In the motor recognition task, we wanted to check whether participants could spontaneously reproduce the learned sequence. Four empty

rectangles were shown on the monitor. Subjects were told to reproduce any finger movement that they could remember. Subjects had time to think, if necessary, before doing the task. They could reproduce the pattern one time. Once they decided to reproduce the movements, they just had to press the buttons in the order they though. There was not a minimum number of trials to be reproduced. The software was programmed to stop after a maximum of 12 inputs, like the number of trials of the deterministic sequence.

We also designed a visual recognition task. It consisted in the observation of 4 visual sequences of 12 positions each. The sequence started with four-empty rectangles that were filled in in a sequence or random order, every 400ms. Unbeknown to the subject, two visual sequences represented the deterministic one and the other two were random sequences. The order of presentation of the four sequences were randomized across participants. Subjects were told to press a button as soon as they could recognize a familiar pattern. Furthermore, the use of the right hand was avoided in this task, due to a potential bias in using the trained hand. Therefore, had to answer with the left hand while the right hand was position behind their back. Once the subject had pressed the button, the sequence stopped.

Measures of Performance

Behavioural parameters

The performance at the SRTT was measured as response time (RT). RT was defined as the time between the onset of a visual cue and the press of the correct button. A filter was applied to extract the data. Specifically, RT lower than 200ms and higher than 2000ms were removed as well as RT longer than 2.7 standard deviation of the participant's mean in each session. Moreover, trials in which an error occurred were removed. Errors were defined as the pressure of the wrong button. Additionally, trials in which the correct response was given soon after the error were also eliminated from the analysis. To better characterize learning of the SRTT we defined a *General performance* index and more specific skills indexes.

The *General performance* index was defined as the difference of RT between the last 50 random trials of the learning session and the last 50 random trials of the final

session (Figure 14). This computation allows to evaluate a general improvement in performance due to mere repetition of the task, independently from the sequences.

To evaluate the learning of a specific skill (that is the learning of the sequence), we computed several indexes: *Skill* was defined as the difference between the mean RT of the last 50 sequential trials and the mean RT of the last 50 random trials. This difference was computed in each session (baseline, learning and final). *Skill* allows to assess the sequence-specific learning in each group and session (Figure 14). To better characterize the potential improvement due to the placebo procedure, we calculated the difference of *Skill* across sessions. Specifically, $\Delta Skill_{(\text{learning-baseline})}$ was obtained by calculating the difference of *Skill* between the learning and baseline sessions. $\Delta Skill_{(\text{final-learning})}$ was defined as the difference of *Skill* between the final and learning sessions. Finally, $\Delta Skill_{(\text{final-baseline})}$ was measured by subtracting *Skill* of the final and baseline session.

To better understand whether the sequential-specific learning changed soon after the placebo application, another index was computed (*Skill**). This index was computed as the difference between the mean RT of the first 50 sequential trials and the mean RT of the first 50 random trials. This difference was computed for the baseline, learning and final sessions.

Furthermore, two more indexes were computed to measure whether the gain (sequential-specific learning) that occurs at the end of each baseline and learning session is maintained or improved after the first and the second placebo application. Specifically, we calculated the difference between *Skill** of the learning session and *Skill* of the baseline session (*Change1)* and between *Skill** of the final session and *Skill* of the learning session (*Change2).* Both *Change1* and *Change2* were also measure in the control group to compare the potential different with both placebo treatments. (Figure 14).

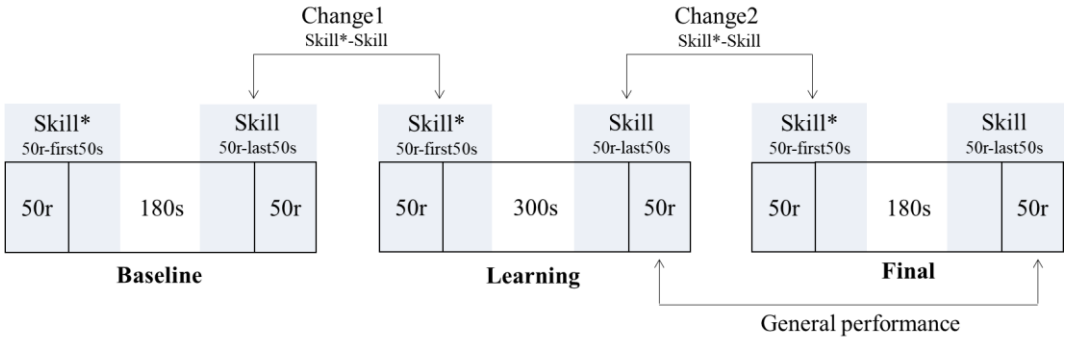

Figure 14. Schematic representation of the indexes computation. The specific-sequence learning in each session was calculate with the index *Skill*, which consisted in the difference between the mean RT of the last 50 sequential trials and the last 50 random trials. *Skill** was also computed and it was defined as the difference between the mean RT of the first 50 sequential trials and the mean RT of the first 50 random trials. The sequence-specific learning soon after the first and the second placebo application was obtained with the computation of *Change1* (difference between *Skill** of the learning session and *Skill* of the baseline session) and *Change2* (difference between *Skill** of the final session and *Skill* of the learning session). Both *Skill* and *Skill** were calculated for each session (baseline, learning and final). Finally, the mere motor improvement due to repetition of the task (*General performance*) was computed as the different between the last 50 random trials of the learning session and the last 50 random trials of the final session. *r* = random; *s* = sequence.

Regarding the motor recognition task, we calculated how many trials the subjects were able to reproduce correctly according to the sequence trials. We considered as correct sequence a minimum of three button presses executed in the same order as in the learned sequence (e.g. 2-4-1). A cluster of three positions remembered in the same order as in the learned sequence was considered as correct even if it occurred in a different position compared to the original sequence of 12 items. The total score was calculated as the sum of the number of trials remembered in the correct sequences (Willingham & Goedert-Eschmann 1999; Brown & Robertson 2007; Önal-Hartman et al., 2012).

With regards to the visual recognition task, we collected two types of information. First, we registered whether the subject recognized the pattern or not. Second, we registered the exact moment in which the sequence was recognized. The recognition of any sequence was scored as 1. A delta (Sequence - Random) was computed to extract the accuracy specific for sequence trials. The maximum score was 2.

Subjective parameters

Subjective parameters were also evaluated throughout the experiment. In all groups, subjective perception of mental and physical fatigue was measured soon after having completed each session (for a total of 3 measurements). Specifically, a 7-points number rating scale (NRS) ranging from -3 (much fatigue) to +3 (much energy), with 0 (the same as before) was used.

The expectation of change in performance was evaluated after each placebo application, for both the Placebo-TENS and the Placebo-tDCS groups. Participants had to judge how much and in which direction they expected the future performance would be compared to baseline on a 7-points NRS ranging from -3 (much worse than at baseline) to +3 (much better than at baseline), with 0 (the same as at baseline). Moreover, the perception of treatment efficacy was measured after the learning and the final session. Subjects had to rate whether the treatment (TENS and tDCS) was effective or not in enhancing the muscle efficiency (for the Placebo-TENS group) or the concentration/attention (for Placebo-tDCS group) on a 10 cm visual analogue scale (VAS) ranging from 0 (not effective at all) to 10 (extremely effective).

Statistical Analysis

A first omnibus analysis on the raw RTs was carried out by means of repeated measure analysis of variance (rmANOVA). Particularly, the factor Group (Placebo-TENS, Placebo-tDCS, Control) was defined as between-subjects factor, and Trial type (Random, Sequence) and Session (Baseline, Learning, Final) were defined as within-subject factors. The *General performance* was analysed by means of one-way ANOVA with the factor Group (Placebo-TENS, Placebo-tDCS, Control) as between-subject factor.

Skill and *Skill** were analysed by means of separate rmANOVA with Group (Placebo-TENS, Placebo-tDCS, Control) as between-subject factor and Session (baseline, learning and final) as within-subject factor. One-way ANOVA was used to analyse the factor Group (Placebo-TENS, Placebo-tDCS, Control) with regard to the delta Skill ($\Delta Skill_{(learning\text{-}baseline)}$, $\Delta Skill_{(final\text{-}learning)}$, $\Delta Skill_{(final\text{-}baseline)}$) and changes (*Change1* and *Change2*).

The motor recognition of the sequence was analysed by means of one-way ANOVA with Group (Placebo-TENS, Placebo-tDCS, Control) as between-subject factor. Similarly, the delta of the visual recognition was measured with one-way ANOVA with Group (Placebo-TENS, Placebo-tDCS, Control) as between-subject factor.

Subjective parameters (physical fatigue and mental fatigue) were analysed by means of rmANOVA with Group (Placebo-TENS, Placebo-tDCS, Control) as between-subjects factor and Session (Baseline, Learning, Final) as within-subjects factor. The expectation scores and the perception of treatment efficacy were analysed by means of rmANOVA with Group (Placebo-TENS, Placebo-tDCS) as between-subjects factor and Session (first treatment application, second treatment application) as within-subjects factor. To check whether the information given to participants about the effect of the treatment was successful in inducing positive expectations and perception of treatment efficacy, one-sample t-test was run to compare the scores of expectation and treatment efficacy against 0 separately for the two applications (first and second) and for both Placebo-TENS and Placebo-tDCS groups.

Bonferroni correction for multiple comparisons was applied when needed and the level of significance was set at $p \leq 0.05$. Data are represented as mean values ±SE.

Results

Behavioural parameters

The omnibus analysis of the RT showed a significant effect of Session ($F_{(2, 86)} = 144.98$, $p < 0.001$) due to faster respond in the final (293.82 ± 2.92ms) than in the baseline (340.02 ± 5.44ms) and learning (307.53 ± 3.63ms) session (for both, $p < 0.001$). Furthermore, subjects significantly reduced the RT in the learning (307.53 ± 3.63ms) compared to the baseline (340.02 ± 5.44ms) session ($p < 0.001$). We also found an effect of the factor Trial type ($F_{(1, 86)} = 129.43$, $p < 0.001$), due to faster performance during the sequence (305.81 ± 3.65ms) than during the random trials (321.77 ± 4.09ms). The interaction Session × Trial type resulted significant ($F_{(2, 86)} = 5.05$, $p = 0.007$). The t-test analysis displayed a lower RT during the sequence trails (330.14 ± 5.60ms) than during the random trial (349.59 ± 5.65ms) in the baseline ($p < 0.001$) session. Similarly, participants during the sequence trails in

both learning (299.23 ± 3.49ms) and final (287.77 ± 2.85ms) were also faster than during the random trials (315.60 ± 4.14ms; 299.70 ± 3.28ms respectively) in both sessions (for both, $p < 0.001$). However, no significant were found effect for the factor Group and for the other interactions ($p > 0.09$). These results suggested that independently of the placebo procedure, there was an overall improvement of RT over time and it resulted better in the sequence than in the random trials (Figure 15).

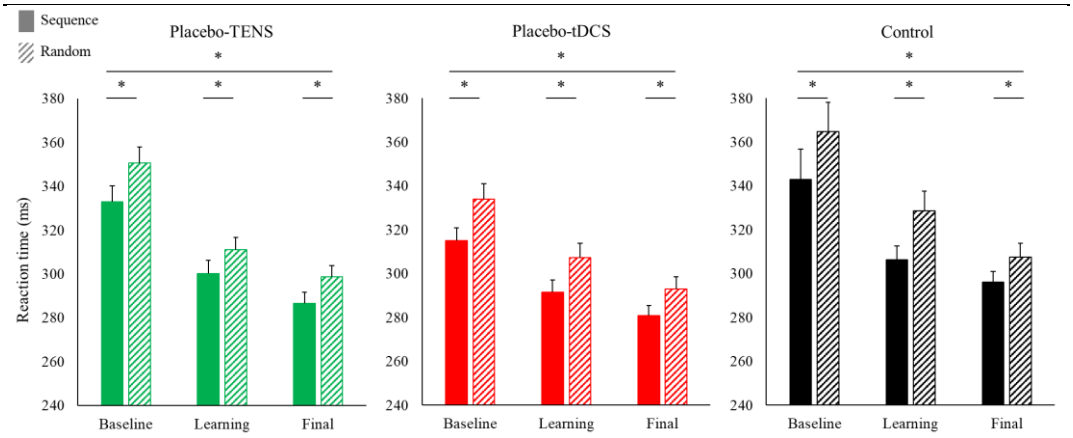

Figure 15. Behavioural performance in the SRTT. Regardless of groups, RT was lower in the final than in the baseline and learning session, as well as in the learning compared to the baseline session. Furthermore, the sequence trails were performed faster compared to the random trials in all groups. Values are expressed as mean ± SE. *p < 0.050.

Regarding the *General performance*, the one-way ANOVA analysis showed no significant difference between groups ($F_{(2, 86)} = 2.88$, $p = 0.061$), suggesting that the improvement due to the aspecific effect of learning was similar for all subjects independently of the group.

The analysis of the *Skill* was found non-significant for any factor ($p > 0.25$) (Figure 15A). In the same way, the *Skill** analysis did not reveal significant effects ($p > 0.078$) (Figure 15B). Moreover, the analysis of delta *Skill* did not show any significant effect ($p > 0.14$).

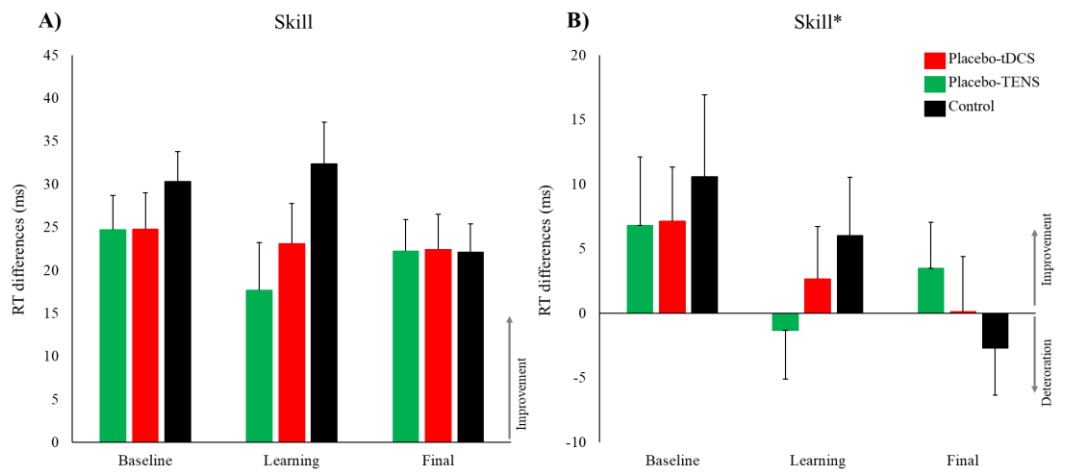

Figure 15. RT differences of the computed indexes. (A) Reaction time differences obtained for *Skill* in each session and group resulted not significant. (B) No significant effects were found between groups and sessions for the index *Skill**. Positive values are indicative of improvement. Values are expressed as mean ± SE.

However, from a qualitative inspection of the data obtained from the $\Delta Skill_{(final-learning)}$ it seems that a difference could emerge between Placebo-TENS and Control group (see Figure 16). By running t-test for independent sample, we discovered indeed that this difference could indicate a trend towards significant ($t_{(57)} = 1.98$, $p = 0.057$). It remains to be checked whether this trend could become significant by increasing the sample.

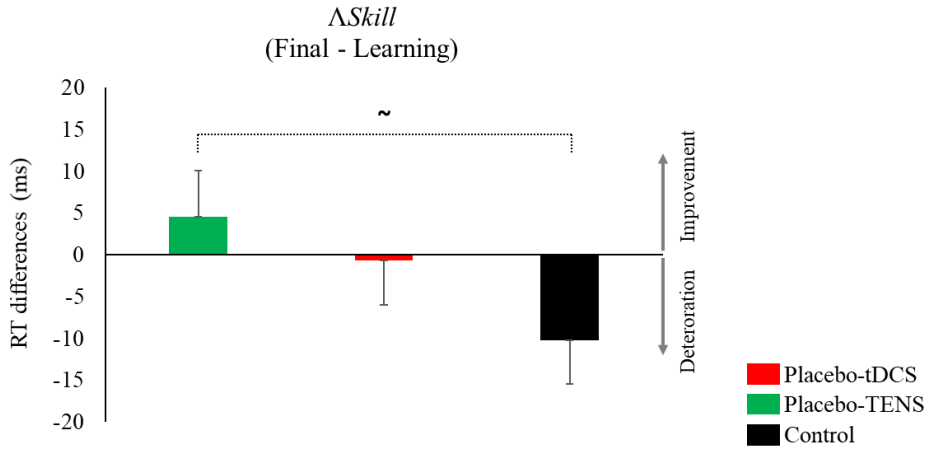

Figure 16. Reaction time differences of $\Delta Skill$. $\Delta Skill_{(final - learning)}$ represents the sequence-specific learning obtained after the second placebo application (difference between *Skill* of the final session and *Skill* of the learning session). Data analysis showed a trend towards

significant between the Placebo-TENS and the Control group, indicating a potential gain of the sequence-specific in the Placebo-TENS compared to the control group. Values are expressed as mean ± SE. ~ p = 0.057.

The *Change* indexes showed that between session gain (faster in sequence than in random trials) is reduced and not different between group was found in *Change1* (*p* = 0.88). However, the analysis of *Change2* was found significant ($F_{(2, 86)}$ = 4.26, *p* = 0.017). The post-hoc analysis revealed that the participants of the Placebo-TENS had group lower reduction of gain (-14.18 ± 4.71ms) than those of the Control (-35.07 ± 4.66ms) group (*p* = 0.003). Thus, the gain that subjects have in *Skill*, which was big in the learning of the control (see Figure 16), showed a strong reduction at the beginning of final (*Change 2*), whereas the Placebo-TENS has also a reduction of this gain when passing from learning to final (*Change2*) (see Figure 16), but this reduction is significantly lower compared to the Control group. This small advantage for the sequence to the random could be related to the second application of TENS. (Figure 17).

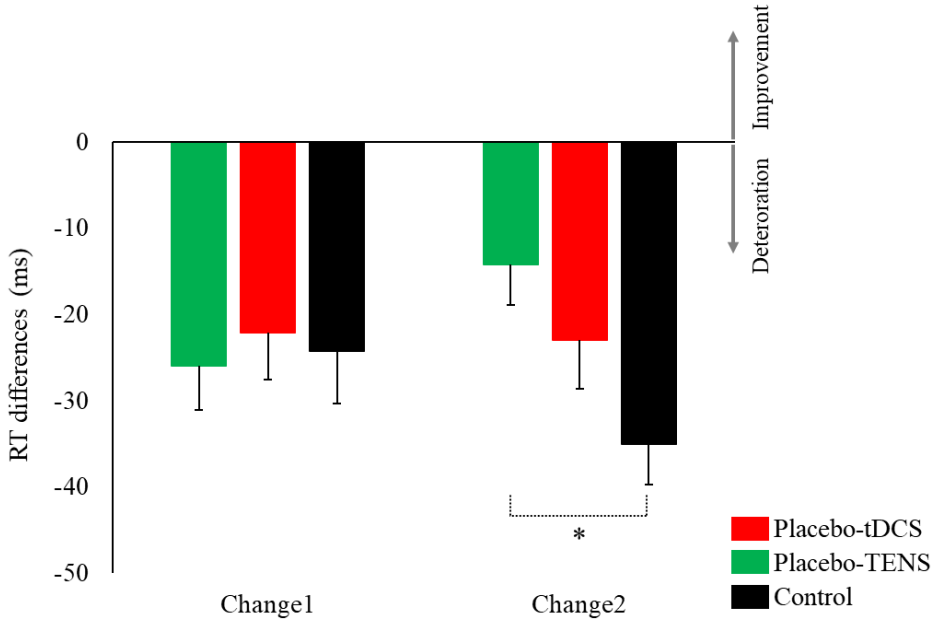

Figure 17. Reaction time differences of *Change*. *Change1* represents the sequence-specific learning obtained soon after the first placebo application (difference between *Skill** of the learning session and *Skill* of the baseline session). *Change2* represents the sequence-specific gain of learning present when passing from the end of the learning session to the beginning of the final session (difference between *Skill** of the final session and *Skill* of the learning session). As shown in the figure, *Change2* was significantly different between the

Placebo-TENS and the Control group, suggesting that the deterioration of the sequence-specific gain was bigger in the control group compared to the Placebo-TENS. Values are expressed as mean ± SE. *p < 0.050.

With regards the motor recognition, the one-way ANOVA analysis found no differences in the number of trials evoked between the three groups ($F_{(2, 42)} = 0.02$, $p = 0.97$). However, the one-way ANOVA of the visual recognition showed a significant effect between groups ($F_{(2, 42)} = 3.51$, $p = 0.034$). Post-hoc comparisons showed that participants from the Placebo-TENS (0.13 ± 0.19) recognized visually less sequence compared to the Control (0.83 ± 0.14) group (p =0.003).

Subjective parameters

The perception of mental fatigue analysis displayed a significant effect of Session ($F_{(2, 86)} = 10.56$, $p < 0.001$). In particular, participants perceived less mental fatigue after the learning (-0.05 ± 0.11) and the final (0.11 ± 0.12) than the baseline (-0.48 ± 0.1) session ($p = 0.008$ and $p < 0.001$, respectively). Moreover, the factor Group was also significant ($F_{(2, 86)} = 4.48$, $p = 0.014$), in which participants of the Placebo-tDCS (0.2 ± 0.13) had in general less mental fatigue than participants of the Placebo-TENS (-0.41 ± 0.11) group ($p = 0.001$). Additionally, the analysis revealed a significant interaction Session × Group effect ($F_{(4, 86)} = 9.62$, $p < 0.001$) in which within the Placebo-tDCS group, participants perceived a lower mental fatigue after the final (0.95 ± 0.21) than the baseline (-0.71 ± 0.18) and learning (0.36 ± 0.2) session ($p < 0.001$ and $p = 0.018$, respectively) and also when comparing the learning (0.36 ± 0.2) to the baseline (-0.71 ± 0.18) session ($p < 0.001$). Interestingly, the participants of the Placebo-tDCS group perceived less mental fatigue after both the learning (0.36 ± 0.2) and final (0.95 ± 0.21) session when compared to the participants of the Placebo-TENS group (-0.43 ± 0.20 and -0.18 ± 0.21) (p <0.001 and p =0.006, respectively). Furthermore, the participants of the Placebo-tDCS showed lower perception of mental fatigue (0.95 ± 0.21) than participants of the Control (-0.43 ± 0.21) group at the final ($p < 0.001$) session (Figure 18A).

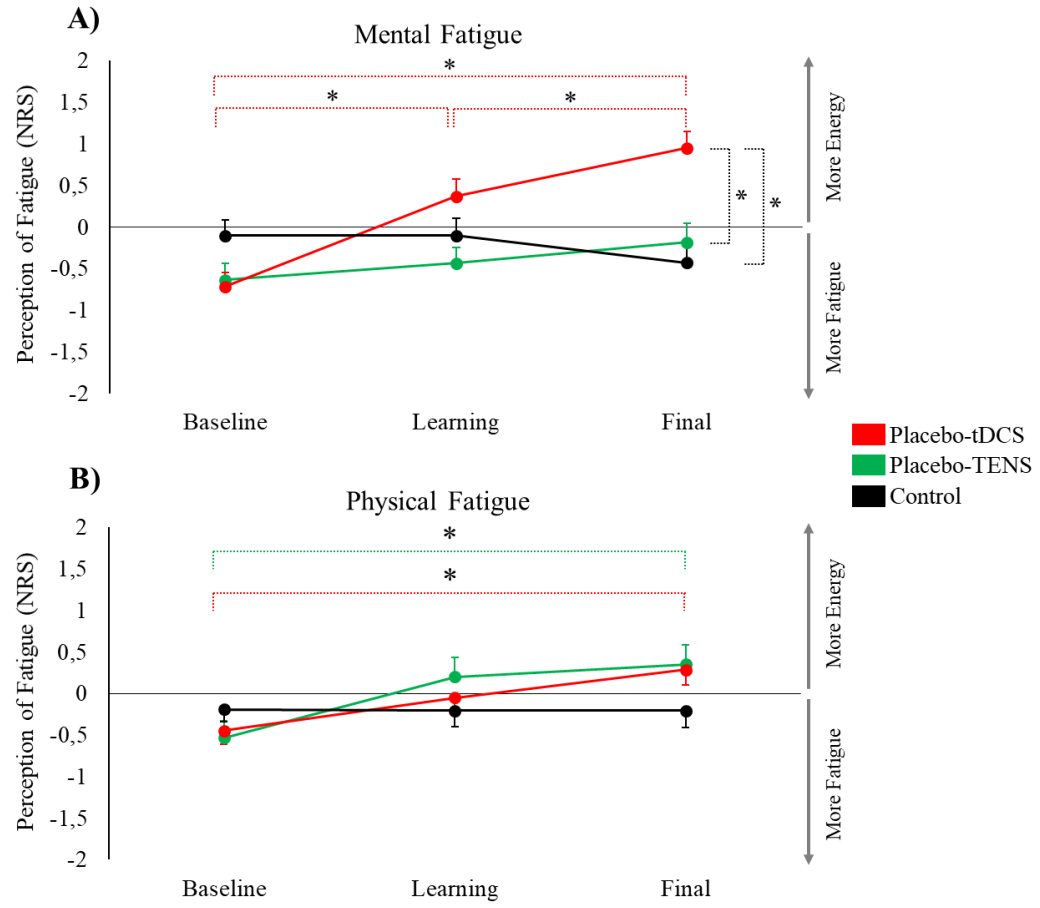

Figure 18. Subjective data of mental and physical fatigue perception. (A) Participants of the Placebo-tDCS perceived less mental fatigue in the learning than in the baseline session. Moreover, they also perceived less mental fatigue in the final compared to both the learning and baseline sessions. Participants of the Placebo-tDCS group perceived significantly less mental fatigue than participants of the Placebo-TENS and Control group in the final session. (B) Participants of both experimental groups (Placebo-tDCS and Placebo-TENS) perceived less physical fatigue in the final compared to the baseline session. Values are expressed as mean ± SE. *p < 0.050.

With regards to the subjective physical fatigue, the rmANOVA displayed an effect on Session ($F_{(2, 86)}$ = 9.39, p < 0.001), showing an overall decrease of physical fatigue after the final (0.14 ± 0.12) and the learning (-0.01 ± 0.11) compared to the baseline (-0.39 ± 0.09) session (p = 0.003 and p = 0.018, respectively). The interaction Session × Group was also significant ($F_{(4, 86)}$ = 9.39, p = 0.028). Post-hoc comparisons showed that participants of the Placebo-TENS perceived lower physical fatigue after the final (0.35 ± 0.23) than the baseline (-0.53 ± 0.20) session (p = 0.027). Similar effect was also found within the Placebo-tDCS group, in which

participants perceived themselves with less physical fatigue in the final (0.28 ± 0.18) than in the baseline (-0.45 ± 0.15) session (p = 0.027). The factor Group, instead, was not significant (p = 0.6) (Figure 18B).

The analysis of expectation scores showed a significant effect of Session ($F_{(1, 58)}$ = 6.20, p = 0.016), due to higher scores after the second (1.38 ± 0.09) than after the first (1.12 ± 0.06) treatment application (p = 0.016) regardless of the experimental group. Instead, the factors Group and interaction were found no significant (p > 0.09). Furthermore, the scores of expectation of change were significantly above 0 in both treatment applications and in both experimental groups (for all comparisons, $t_{(29)}$ > 9.10, p < 0.001) suggesting that participants of both Placebo-TENS and Placebo-tDCS expected an improvement in their performance (Figure 19A).

With regards to the perception of treatment efficacy, rmANOVA analysis revealed a significant effect of Session ($F_{(1, 58)}$ = 24.43, p < 0.001), because of higher perception of treatment efficacy after the final (6.28 ± 0.30) than after the second (5.13 ± 0.27) session (p < 0.001). However, Group (p = 0.33) and Session × Group (p = 0.35) were not significant. Moreover, scores of treatment efficacy revealed a significant difference from 0 both in the first and in the second application of the placebo treatment and in both experimental groups (for all comparisons, $t_{(29)}$ > 11.28, p < 0.001), indicating that participants believed in the effect of TENS and tDCS (Figure 19B).

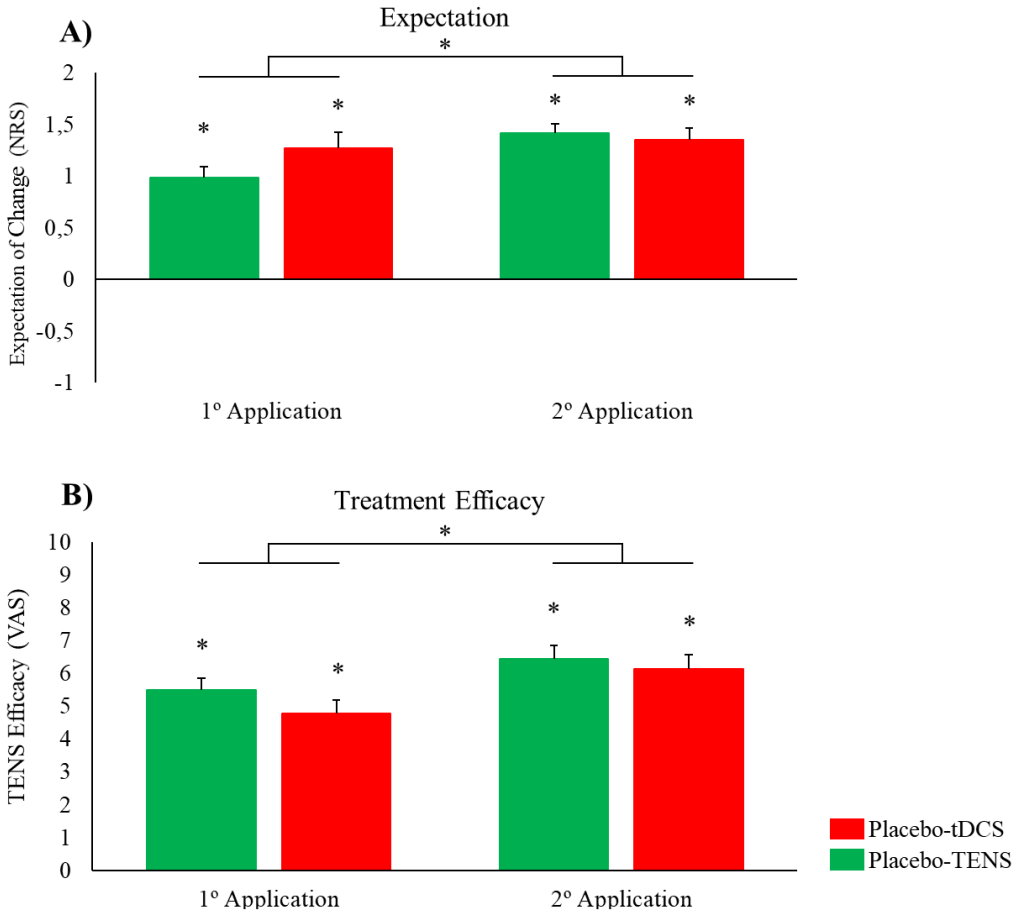

Figure 19. Subjective data of expectation and treatment efficacy. (A) Subjects of both experimental groups (Placebo-tDCS and Placebo-TENS) expected a better performance after both treatment applications. Moreover, in both groups the expectation of change was significantly higher after the second than the first treatment application. (B) Participants of the Placebo-tDCS and Placebo-TENS group perceived the treatments as more effective after the second than the first application. Furthermore, both experimental groups perceived a significant positive effect of tDCS and TENS both after the second and the first application. * above the bars indicate differences from 0. Values are expressed as mean ± SE. *$p < 0.050$.

Discussion

The aim of this study was to investigate whether the placebo effect could be induced in a motor sequence learning task, like the serial reaction time task (SRTT). Since the task involves both motor and cognitive aspects, we were also specifically interested in exploring whether a placebo procedure directed toward the improvement of motor functions (Placebo-TENS) or of cognitive functions

(Placebo-tDCS) could differently contribute to the motor sequence learning. Our results reveal that while the subjective perception of mental and physical fatigue could be clearly influenced by the placebo procedures, the behavioural parameters of performance were not strongly modulated by the placebo effect. More precisely, the Placebo-tDCS group perceived less mental and physical in the final than in the baseline session and the Placebo-TENS group perceived less physical fatigue in the final than in the baseline session. This may indicate that while the tDCS placebo procedure directed toward the cognitive functions (i.e., concentration and attention) could impact on both mental and physical fatigue, the TENS procedure directed toward the motor functions (i.e., muscle activity) could impact only on physical fatigue. Despite these clear subjective results, the behavioural findings were not so clear-cut. We found that all the groups improved in the SRTT. With regard to the sequence-specific learning a pattern seems to emerge that indicates a better learning in the Placebo-TENS group compared to the other groups, although caution should be taken in interpreting this finding, as discuss in detail below.

Placebo effects increase perceived performance but not the behavioural performance

In this study, we tried to investigate whether a placebo procedure could modulate motor learning depending on the specificity of the placebo procedure applied. Regardless of the placebo procedure applied, our results show higher expectation of change in the second than in the first treatment application. Moreover, we also observe a robust perception of treatment efficacy in both experimental groups. These findings suggest that the two placebo procedures were successful in inducing perception of treatment efficacy and positive expectation. Furthermore, these findings are in line with previous studies showing that both TENS and sham tDCS are suitable treatments to induce positive expectations (Fiorio et al., 2018; Turi et al., 2018).

Despite the positive expectations and the perception of treatment efficacy, the behavioral data did not show a clear placebo effect on motor sequence learning. This could suggest that, differently from other motor functions, like force, speed or resistance to fatigue (Beedie et al., 2006; Beedie et al., 2008; Fiorio et al., 2014;

Piedimonte et al., 2015; Pollo et al., 2011) motor sequence learning is less permeable to the positive influence of the placebo effect. The SRTT is a compound of motor and cognitive components (Janacsek & Nemeth, 2012; Kaufman et al., 2010) and this complex feature could make it difficult to investigate the effects of placebo on learning. Previous studies on other types of learning found a placebo effect on performance. More precisely, a study by Colagiuri et al., (2011) tested whether expectation induced via an instructional manipulation could modulate implicit learning in a visual task. Participants of the placebo group received a container with a cotton pad impregnated with an odor (bubblegum, actually inert) along with positive information indicating that the odor could improve cognitive performance by enhancing attention. A similar procedure was adopted for the control and nocebo groups, but with neutral or negative information, respectively. Interestingly, subjects of the placebo group showed a reduction in the reaction time compared to the control and nocebo group, suggesting a better implicit learning (Colagiuri et al., 2011). Differently from that study, in which the placebo odor was present online throughout the performance of the task, in our case we applied the placebo treatment offline, that is before the execution of the task. Hence, one possible explanation for our finding could be related to this methodological choice. Moreover, in our case, the task was visuo-motor, not only visual as in Colagiuri et al. (2011), thus adding a further element of difference between the two studies.

Other studies did show an improvement of cognitive functions (i.e. reward learning and probabilistic learning) due to a placebo intervention with sham tDCS (Turi et al., 2017; Turi et al., 2018). It is worth noting that differently from our study, the Authors used a placebo procedure consisting in the application of both verbal suggestion and conditioning. This suggests that the application of a conditioning procedure could be an important factor to induce behavioral placebo effects in the context of learning. So, it is reasonable to think that verbal suggestion and conditioning could induce a stronger placebo effect able to modulate the behavioral parameters of the SRTT. Further studies should be conducted to deeply investigate whether a placebo procedure could improve an implicit SRTT, as well as, considering the potential effect of an “online” inert stimulation, similar to online tDCS stimulation.

Placebo effects reduce physical and mental fatigue

More interestingly, our findings demonstrate that placebo procedures focused on both the cognitive functions, like concentration and attention (Placebo-tDCS) and on the motor function, like muscle activity (Placebo-TENS) were successful in inducing a reduction of the perception of physical fatigue, proving that our protocol was suitable to reduce physical fatigue, like other placebo studies on motor performance (Beedie et al., 2008; Clark et al., 2000; Pollo et al., 2008). It is also remarkable that not only the TENS treatment, which was applied directly to the hand involved in the task improved the perception of physical fatigue, but also the sham tDCS treatment, suggesting a potential role of the placebo sham tDCS as an ergogenic aid for motor performance. Sham tDCS is considered as a reliable control stimulation procedure since it does not induce cortical modulation and it is not distinguishable from active tDCS (Gandiga et al., 2006). This potential and new characteristic of sham tDCS in the placebo effect in the motor domain could open new investigations in the motor field, for instance to reduce the perception of physical fatigue in contexts in which movement repetition is often a requisite, like in motor training, motor learning and motor rehabilitation.

Other recent studies converge in indicating that the application of sham tDCS can induce a placebo effect (Turi et al., 2017; Turi et al., 2018). All these evidences point out to the possibility of using sham tDCS during the performance of motor training, motor learning or motor rehabilitation protocols. This is also possible because tDCS can be applied in "online" mode, that is during a motor or cognitive task (Thair et al., 2017).

Participants of the Placebo-tDCS group showed a significant reduction of mental fatigue across sessions and perceived less mental fatigue than both the Control and the Placebo-TENS group at the end of the experiment. This finding suggests that a placebo procedure that focuses on cognitive functions (increase of attention and concentration) can lead to a significant improvement of mental fatigue perception. Mental fatigue can be induced by prolonged demanding cognitive or mental activities (Mizuno et al., 2011; Pageaux & Lepers, 2018) and seems to have a relevant role in the performance of certain physical activities (MacMahon et al.,

2014; Marcora et al., 2009; Pageaux et al., 2014; Van Cutsem et al., 2017). A previous study found that the decrease of performance in a time-to-exhaustion cycling task was due to mental fatigue (Marcora et al., 2009). In another study the time to complete self-paced running protocol was longer when participants reported mental fatigue (MacMahon et al., 2014; Pageaux et al., 2014). Previous studies on placebo effect in the motor domain have already shown the benefits of placebo in endurance exercise (Benedetti et al., 2007; Carlino et al., 2014; Carlino et al., 2016; Clark et al., 2000; Piedimonte et al., 2015; Pollo et al., 2008). Our study suggests that maybe an underlying mechanism could be found in the perception of mental fatigue associated with endurance exercise.

More remarkably, scientists have shown how mental fatigue can also impair the performance of some motor skills or sport-technical skills (Duncan et al., 2015; Pageaux & Lepers, 2018; Rozand et al., 2015; Smith et al., 2016; Veness et al., 2017). For instance, in a crossover study by Rozand et al. (2015), participants had to perform a task consisting of a point-to-point arm movement as fast and accurately as possible before and after a 90min of sustained cognitive task (experimental condition) or after having watched a documentary for 90min (control condition). Authors found that the movement was slower when subjects were mentally fatigued (experimental condition) than when they were not mentally fatigued (control condition). Moreover, Duncan et al. (2015) investigated whether the induction of mental fatigue could modulate the performance of a manual dexterity test in a crossover study. In particular, participants underwent a manual dexterity task before and after 40min of either a continuous cognitive task (inducing mental fatigue) or a passive neutral observation procedure. Authors found that after the induction of mental fatigue, participants needed a longer time to conclude the manual dexterity test (Duncan et al., 2015). The presence of mental fatigue can also have negative effects in sport-technical skills. For instance, mental fatigue can impair the passing accuracy and the speed of shooting in football players (Smith et al., 2016). These evidences show the potential role of mental fatigue in the performance of motor performance and skills. Up-to-now, no study has tackled the effect of mental fatigue on motor skill learning. According to the studies commented above, it is plausible to hypothesize that motor skills learning in which the mental demand is high (sport-

technical skill or medical-technical skills) or the training is prolonged in time (i.e. massed practice in rehabilitation programs) could be affected by the presence of mental fatigue. In these cases, the fact that we could reduce the subjective perception of mental fatigue by a placebo procedure might lead us to suggest that the application of sham tDCS along with information on the beneficial effects on attention and concentration could be helpful to the performance of a motor skill training or motor rehabilitation program. Thus, extending the period of learning thanks to the reduction of mental fatigue could potentially lead to learn some skills or re-acquired lost movements in a shorter period of time. However, caution should be taken, and additional research may be necessary.

GENERAL CONCLUSION

My interest to investigate the placebo effect in the motor domain is motivated by my own scientific curiosity and by the potential translational impact of the motor placebo effect in sports and pathology. Due to the fact that very little is known about this phenomenon, it is mandatory to clarify some questions before moving to sport or clinical studies. To do so, we decided to investigate the placebo effect at two levels: one, regarding to the neural correlates of the placebo effect in the motor domain; the other referring to the type of motor functions that could be influenced by the placebo effect. Therefore, during the description of my thesis, I have tried to show to the reader the studies and experiments that we carried out to fill in the gaps of knowledge that were present with regards to the placebo effect in the motor domain at both levels.

The neural correlates of the motor placebo effect in healthy participants have been related to brain areas involved in movement execution and preparation (Fiorio et al., 2014, Piedimonte et al., 2015). In the *Part II* of this thesis, we attempted to enlarge this knowledge by investigating the role of high-order frontal areas, like the dlPFC. Our finding demonstrated that active tDCS (both anodal and cathodal tDCS) over the left dlPFC could block the placebo-induced increase of force found in placebo-responders when tDCS was inactive. This effect was detected only when the placebo procedure consisted of expectation alone. However, tDCS showed no effect on the placebo-induced increase of force when a conditioning procedure was adopted. This finding may indicate that the left dlPFC plays a role in the motor placebo effect induced by verbal suggestion alone. In this study, we demonstrated for the first time that the left dlPFC could be involved in the expectation-induced enhancement of force, thus enlarging the existing knowledge on the neural correlates of the placebo effect in the motor domain in healthy participants.

Regarding the behavioral investigation of the placebo effect in the motor domain, several studies have shown the influence of placebo on force production, movement speed and resistance to fatigue (Beedie et al., 2009; Fiorio et al., 2018; Pollo et al., 2011). However, these studies were principally focussed on sport performance rather than motor functions present in daily life activities, like the control of balance

or motor sequence learning. Thus, in the *Part III* of the thesis, we have explored whether a placebo procedure could improve balance control and motor sequence learning in healthy participants.

With regards to the first study in which we evaluated the effect of placebo in balance control, we have given the first behavioural evidence that a placebo procedure in the motor domain can also improve balance control and perception of stability. These findings could have significant translational application for clinical populations (like for instance Parkinson's disease) and for the elderly, in which balance disturbances increase the risk of falls with a resulting negative impact on the quality of life. Furthermore, improving balance through a placebo procedure could also have a beneficial impact on gait disorders in which the pharmacological treatment is often not effective. In our study, we did not investigate the potential neural correlates of the placebo effect in balance control. Consequently, future neurophysiological investigations will be needed to reveal the exact mechanisms underpinning the placebo-induced enhancement of balance.

On the second study performed in the *Part III*, we have investigated whether the placebo effect could modulate a motor sequence learning by two placebo procedures; one focused on cognitive functions, like concentration and attention (sham tDCS); and the other focused on the motor function, like muscle activity (TENS). In our experiment, we did not find higher improvement of motor sequence learning in neither Placebo-TENS nor Placebo-tDCS when compared to the control group. Remarkably, our subjective results, instead, have found a reduction of the perceived fatigue throughout the sessions in both placebo groups. Namely, a placebo procedure on the motor function (TENS) can reduce the perception of physical fatigue during the SRTT. Interestingly, a placebo procedure addressing the cognitive function (sham tDCS) can decrease not only the perception of physical fatigue, but also of mental fatigue. The fact that this placebo procedure (sham tDCS) has demonstrated its relevant role in reducing both fatigue perception (mental and physical) in a motor sequence learning task, opens the possibility to explore new strategies to investigate the placebo effect in the motor domain. Specifically, sham tDCS could be applied as an "online" placebo treatment and be implemented in motor training or motor rehabilitation as well. Further studies on consolidation or

longer period of motor skill learning should be conducted to gain a better understanding of these effects.

To summarize, we have been able to expand the actual knowledge about the placebo effect in the motor domain regarding both levels of study, the neural correlates and the behavioral influences of this phenomenon, thus, showing that the left dlPFC could be involved in the placebo effect in the motor domain, particularly when the placebo effect is induced by verbal suggestion alone. Moreover, the control of balance could be modulated by the induction of positive expectation through verbal suggestion. Lastly, a placebo procedure focused on cognitive function could reduce the perception of both mental and physical fatigue in a repetitive motor task.

Although we have contributed to enlarge the knowledge about the placebo effect in the motor domain, many questions remain unanswered. Thus, further studies should be performed to unveil the mechanisms of the placebo effect in the motor domain, not only in healthy population, but also in people affected by movement or gait disorders, in which the placebo effect could potentially improve motor performance and the fatigue perceived during a repetitive motor task.

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LIST OF PUBLICATIONS

Villa-Sánchez, B., Emadi Andani, M., & Fiorio, M. (2018). The role of the dorsolateral prefrontal cortex in the motor placebo effect. *Eur J Neurosci*., 48(11), 3410–3425.

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