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# SHAPING THE BRAIN WITH ELECTRICITY.

# MODULATING CORTICAL EXCITABILITY AND PLASTICITY WITH TRANSCRANIAL RANDOM NOISE STIMULATION

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La mia vita?

Un rapporto assai discutibile, affatto raro,

addirittura imperdonabile, sempre con te.

Anche stamattina, vedi?, s'alza il sole vermiglio

E subito m'acceca.

Ecco, adesso tutto t'assomiglia.

**Anna Merlotti**
**(Mia Nonna)**

Tutto fu ambito e tutto fu tentato.

Quel che non fu fatto io lo sognai;

e tanto era l'ardore che il sogno

eguagliò l'atto.

**Gabriele D'annunzio**

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## GLOSSARY OF ABBREVIATION

**2IFC**: Two-Interval Forced Choice

**AMPA**: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid

**a-tDCS**: anodal transcranial Direct Current Stimulation

**BA**: Brodmann Area

**CNS**: Central Nervous System

**CS**: Contrast Sensitivity

**CSF**: Contrast Sensitivity Function

**cTBS**: Continuous Theta Burst Stimulation

**c-tDCS**: cathodal transcranial Direct Current Stimulation

**DLPFC**: Dorsolateral Prefrontal Cortex

**EF**: Executive Functions

**EMG**: Electromyography

**EPSP**: Excitatory Postsynaptic Potential

**FA**: False Alarms

**FEF**: Frontal Eye Field

**fMRI**: functional Magnetic Resonance Imaging

**hf-tRNS**: high-frequency Transcranial Random Noise Stimulation

**HIT**: Correct Response

**IFG**: Inferior Frontal Gyrus

**iTBS**: Intermittent Theta Burst Stimulation

**lf-tRNS**: low-frequency transcranial Random Noise Stimulation

**LTD**: Long-Term Depression

**LTP**: Long-Term Potentiation

**M1**: Primary Motor Cortex

**MEP**: Motor Evoked Potential

**NIBS**: Non-Invasive Brain Stimulation

**NMDA**: N-Methyl-D-Aspartate

**PFC**: Prefrontal Cortex

**PL**: Perceptual Learning

**RT(s)**: Reaction Time(s)

**SMA**: Supplementary Motor Area

**SR**: Stochastic Resonance

**sRT**: Simple Reaction Time (task)

**tACS**: Transcranial Alternating Current Stimulation

**TBS**: Theta Burst Stimulation

**tES**: Transcranial Electrical Stimulation

**TMS**: Transcranial Magnetic Stimulation

**tRNS**: transcranial Random Noise Stimulation

**V1**: Primary Visual Cortex

**VA**: Visual Acuity

**VLPFC**: Ventrolateral Prefrontal Cortex

# SYNOPSIS

The development of new technologies such as non-invasive brain stimulation (NIBS) methods, allowing a safe modulation of neural activity by altering neuronal excitability and neuroplasticity (Fritsch et al., 2010; Stagg et al., 2009), has become a new frontier in cognitive neuroscience. Their popularity has sharply increased in the last decade, both in basic research and in clinical settings.

The possibility to target specific brain areas painlessly and to interact with the behaviour non-invasively raised a variety of potential investigations. In cognitive neuroscience, it allows investigating the causal relationship between the activity of specific cortical areas with the behaviour or the associated cognitive functions.

Although the most used technique is transcranial magnetic stimulation (TMS), transcranial electrical stimulation (tES) has recently established its role as a promising tool for influencing brain functions, and even for enhancing cognitive, perceptual or motor performances, with potential benefits for pathological conditions. While transcranial direct current stimulation (tDCS) is the most investigated tES techniques (Santarnecchi et al., 2015), more recently, interest has developed in transcranial random noise stimulation (tRNS), which consists of the application of alternating current over the cortex at random frequencies.

Many studies investigated the effect of tRNS on cortical excitability by targeting the motor cortex and measuring TMS-induced motor-evoked potentials (MEPs) (Fritsch et al., 2010; Terney et al., 2008). By applying high-frequency tRNS compared to other tES techniques over motor areas, it was found a larger increase in neural excitability

(Inukai et al., 2016; Moliadze et al., 2014) and an enhancement in motor and visuomotor learning (Prichard et al., 2014; Saiote et al., 2013).

tRNS has also been explored within the visual domain (Pirulli et al., 2013), resulting in being effective, when combined with a perceptual training, in improving visual function in healthy people (Fertonani et al., 2011), in people with myopia (Camilleri et al., 2014), and in clinical population such as people with amblyopia (Campana et al., 2014; Moret et al., 2018; Saiote et al., 2013) and cortical blindness (Herpich et al., 2019).

Evidence of the effectiveness in modifying cortical plasticity and brain excitability are growing, making these tools object of interest in combination with cognitive and behavioural interventions to potentiate their outcomes (Brem et al., 2018; Cappelletti et al., 2013; Snowball et al., 2013).

This thesis concentrates on the investigation of tRNS as a technique to boost brain functioning and to promote plastic effects that could increase beneficial compensatory activity or reduce inefficient neural activity, basing on the favourable but still few results available. In particular, the focus is to investigate the neural plasticity of the human brain using tRNS independently and combined with behavioural training. More in detail, this thesis aims are: first, the exploration of whether and how tRNS is able to modulate neural excitability and to boost neuroplasticity in different cortical substrates; second, to find out which methodological approach induce larger and longer-lasting modulation of neural excitability and plasticity effects; and the last but not the least for importance, to improve the scientific knowledge to promote the

investigation of new therapeutic procedures to extend the use of these promising non-invasive techniques to a broader clinical setting.

The first part of this manuscript provides an introductory overview of the recent literature regarding brain plasticity and NIBS, focusing more on tES, specifically tRNS and its application in motor, visual and cognitive domains. The second part describes the experimental studies I undertook.

More in detail, chapter 2 describes how several stimulation parameters may affect tES effects; specifically, it is referred to tRNS, with the focus on the effect of the high-frequency band on primary motor cortex (M1) excitability. Only a few studies investigated the effect of the frequency band in different fields. In the motor domain, Terney and colleagues (2008) found a consistent excitability increase after 10 minutes of hf-tRNS (but no effect of lf-tRNS) lasting up to one hour, as measured through both physiological measures, motor evoked potentials (MEPs), and behavioural tasks. This result was fairly reproduced by Laczò and colleagues (2014): after 10 minutes of hf-tRNS over M1, they found an increase in excitability for the following 40 minutes after stimulation (Laczò et al., 2014). In the visual domain, Campana and colleagues (Campana et al., 2016) found that, while high-frequency tRNS (hf-tRNS) delivered bilaterally over visual areas V5/MT reduced the duration of motion adaptation, low-frequency tRNS (lf-tRNS) increased it.

Here, we investigate the effects of hf-tRNS on neural excitability, by splitting the whole high-frequency band into a lower and a higher half. The findings of this study suggest that a wide range, compared to the reduced frequency bands, is required to induce a cortical excitability increase (Moret et al., 2019).

Chapter 3 describes an innovative experimental protocol consisting of tRNS coupled with perceptual training aimed to improve visual function in patients with amblyopia. Amblyopia is a developmental disorder of spatial vision, and it is the most frequent cause of vision loss in children occurring in about 2–4% of the population. It manifests as a reduction of visual functions in one or both eyes, despite optimum optical correction and the absence of overt pathology of the visual system (Ciuffreda et al., 1991). Recent studies by Levi and Li (2009) and Camilleri et al. (2014), showed that the lateral masking paradigm, consisting in a contrast detection of a central Gabor stimulus (a sinusoidal grating in a Gaussian envelope) flanked by two high contrast Gabors, was able to produce the most significant improvement ratio on both visual acuity and contrast sensitivity measurements (Polat et al., 2004; Polat and Sagi, 1993).

In light of the results found in the previous study, which showed that the entire high-frequency band induced a more significant increase in cortical excitability, in this second study, we used the whole high-frequency band. Given the potential of transcranial electrical stimulation in boosting the effects of perceptual training when applied online in healthy subject, and considering the larger effect induced using hf-tRNS (Fertonani et al., 2011), we combined eight sessions of hf-tRNS with a lateral masking training to improve visual functions in subjects with amblyopia. TRNS has been shown useful to reduce the number of training sessions needed to obtain a noteworthy long-lasting improvement of visual functions in the amblyopic eye (Moret et al., 2018).

Chapter 4 describes the third study of this project, which involved, in addition to tRNS, an exergame training (physical exercise combined with a videogame) chosen as a

potential training tool for healthy young adults to improve the motor response speed and the response time when inhibition is required. The chosen off-the-shelf exergame (Dr Kawashima: Exercises for the Mind and the Body) takes advantage of a technology that detects body movements; it is considered a type of dynamic videogame since it requires physical exercise. This exergame specifically trains motor and cognitive functions such as planning, execution, monitoring and inhibition, the so-called executive functions (EF), associated with the activation of the prefrontal cortex activation (PFC), the area targeted with tRNS.

We focused our interest on how the exergame training combined with tRNS may affect executive control, behavioural inhibition and processing speed. The results showed an improvement in performance when these techniques have been used individually, with no advantage of using them jointly. Interestingly, the exergame training led to an improvement of simple reaction time, while the tRNS showed its efficacy in a higher demanding task, the Go/NoGo, which also requires inhibition control, with faster performance in go trials.

The conclusion (Chapter 5), encompassing the results obtained in all the studies presented, provides a final discussion regarding the advantages and disadvantages of the chosen designs, the safety of the techniques used and the future directions.

This manuscript aims to contribute to the understanding of the mechanisms of action of tRNS in modulating neural excitability and boosting brain plasticity and offers new insights into the combined approach of tRNS and behavioural training.

Future directions include creating well-calibrated protocols exploiting NIBS and behavioural training, in order to improve, compensate and recovery our abilities toward new perspective of treatment.

# CHAPTER 1. BRAIN PLASTICITY AND NON-INVASIVE BRAIN STIMULATION

## 1.1. Brain plasticity

Brain plasticity is the expression of the extraordinary ability of the human brain to reorganise itself by modifying its structure and its function to adapt to changing demands over a lifetime (Citri & Malenka, 2008; Pascual-Leone, Amedi, Fregni, & Merabet, 2005).

In 1890, William James was the first to use the word "plasticity" applied to behaviour writing: ”Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity” in The Principles of Psychology (James, 2018). However, years later was the neuroscientist Jerzy Konorski the first scientist to use the term *neural plasticity* (Konorski, 1948; LeDoux, 2002; Livingston, 1966).

Brain plasticity, neural plasticity or neuroplasticity is the brain’s characteristic of being plastic, in other words, the ability of the nervous system, to shape and continuously remodel itself, in relation to the stimuli it receives. These spontaneous changes not only are necessary for healthy postnatal development, but they occur in response to different environmental conditions and, also to compensate for brain damages (Møller, 2006).

It is well known that the brain’s plasticity is related to age. First, Wiesel and Hubel (1963) through visual deprivation experiments in kittens, and later Kinkle and colleagues (2001) investigating the auditory cortex, demonstrated that an immature

nervous system has a greater plasticity than a mature nervous system (Klinke, Hartmann, Heid, Tillein, & Kral, 2001; Wiesel & Hubel, 1963). This concept is strictly connected to what in developmental psychology and biology is named "critical period", a short and defined time window during which the presence (and the type) or absence of environmental inputs causes irreversible modifications in brain structure and function. Similarly, during the so-called "sensitive period", the nervous system more easily learns with a greater impact on its architecture, inducing certain patterns of connectivity to become highly preferred (Knudsen, 2004; Lamendella, 1977; Oyama, 1979).

Structural and synaptic plasticity

Two types of neuroplasticity can be identified: structural plasticity and synaptic plasticity. Structural plasticity refers to changes in neuronal morphology (axons, dendrites, and dendritic spines). It consists of the creation and suppression of synapses, and genesis of new neurons, axon terminals and dendrites branches. This process is called sprouting. Like the creation, also the disruption may occur and exiting connections may be lost likely due to the inactivity. This process is called pruning if regards synapses, and apoptosis when is related to parts of the neurons or the whole-body removal. The pruning and apoptosis processes could be a functional response of the brain when the synapses or the neurons are not utilised because no effective. For instance, it occurs in the course of brain development by eliminating all unnecessary nerve cells or, because of the inability of the input to reach the threshold of the target

neuron, or the amount of neurotransmitter released is not sufficient to generate a response. This in turns weakens the connection until it is lost (Møller, 2006).

Synaptic plasticity refers to changes in synaptic activity, leading to modifications of synaptic efficacy and of the behaviour. For instance, it refers to the ability to transfer functions from a damaged area of the brain to other undamaged areas. This last process may occur in response to injury (Pascual-Leone et al., 2011).

The main discriminating factors of synaptic plasticity are "direction", and time persistence of the modification of the connection strength. Following this classification, it is, therefore, possible to distinguish synaptic plasticity in potentiation and depression, and in short-term and long-term plasticity, respectively. Both short-term and long-term potentiation or depression may occur.

Short-term plasticity

Short-term plasticity refers to the dynamic changes in synaptic strength, from milliseconds to minutes lasting up to 30 minutes after which it returns ”normal”.

Cortical excitability can be considered as a form of short-term plasticity. It is defined as the strength of the response of cortical neurons to a given stimulation, and it reflects neuron reactivity and response specificity.

It represents a fundamental characteristic of the brain functioning; it originates from numerous forms of stimulation and, in humans, cortical excitability can be studied through transcranial magnetic stimulation, a non-invasive tool able to modulate cortical activity.

Long-term plasticity

Long-term plasticity persists for a long time, even for hours, days, weeks or years and, in this case, complex biochemical and cellular mechanisms are activated resulting in consolidated changes at the level of brain circuits.

Within the long-term plasticity a fundamental distinction, especially to investigate the effect of a treatment, is between long-term potentiation (LTP) and long-term depression (LTD) (Bliss & Cooke, 2011; Cooke & Bliss, 2006).

LTP and LTD are persisting modification of synaptic strength. This concept introduced by Donald Hebb in 1949 in his book "The organization of behavior" (Hebb, 1949), summarised as "Neurons that fire together, wire together" (Bear, Cooper, & Ebner, 1987; Stent, 1973) is referred to the increased weight between two neurons only when the two neurons are activated simultaneously. Conversely, the strength is reduced if they are activated separately. This causation which requires temporal precedence, today is also known as spike-timing-dependent plasticity, which explains why some synapses become more efficient after being stimulated (Caporale & Dan, 2008).

LTP consists of a form of activity-dependent plasticity which results in a persistent enhancement of synaptic transmission. LTP is input-specific since changes can be induced at one set of synapses on a single cell without influencing other synapses.

During the opposing process of LTP, named LTD, the synaptic connections between neurons become weaker, so the efficacy of synaptic transmission.

The mechanisms behind the LTP and LTD are various and not completely understood. One known mechanism involves glutamate receptors, specifically, NMDA (N-Methyl-D-aspartate) receptors. Only after postsynaptic neuronal depolarisation by multiple

presynaptic inputs (from one neuron or multiple neurons), in quick succession, the magnesium ions that usually block NMDA receptors are removed through an electric repulsion process. Hence, NMDA receptors are immediately able to start the synaptic communication in two ways: the first, transporting sodium and potassium ions that activate depolarisation just like the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, the second allowing calcium ions pass into the postsynaptic cell. Then, this inflow of ions initiates a signalling cascade that causes the increase of a different type of glutamate receptor, AMPA receptors, in the postsynaptic membrane. Once the AMPA receptors are activated, more positive ions enter the cell. Consequently, when the next time glutamate is released from the presynaptic membrane, it will have a larger excitatory postsynaptic potential (EPSP) because more positive ions inflow is allowed thank to the binding of glutamate to the AMPA receptors. Again, the addition of further AMPA receptors strengthens the synapse, so the postsynaptic neuron is more likely to fire in response to presynaptic neurotransmitter release.

On the contrary, during LTD, calcium that enters via NMDA receptors induces the removal of AMPA receptors from the postsynaptic membrane through a different signalling cascade. As a result, the postsynaptic neuron is less responsive to the glutamate released from the presynaptic neuron. The weakening process may lead to the pruning of idle synapses, leaving only the salient connections strengthened by LTP (Figure1.1).

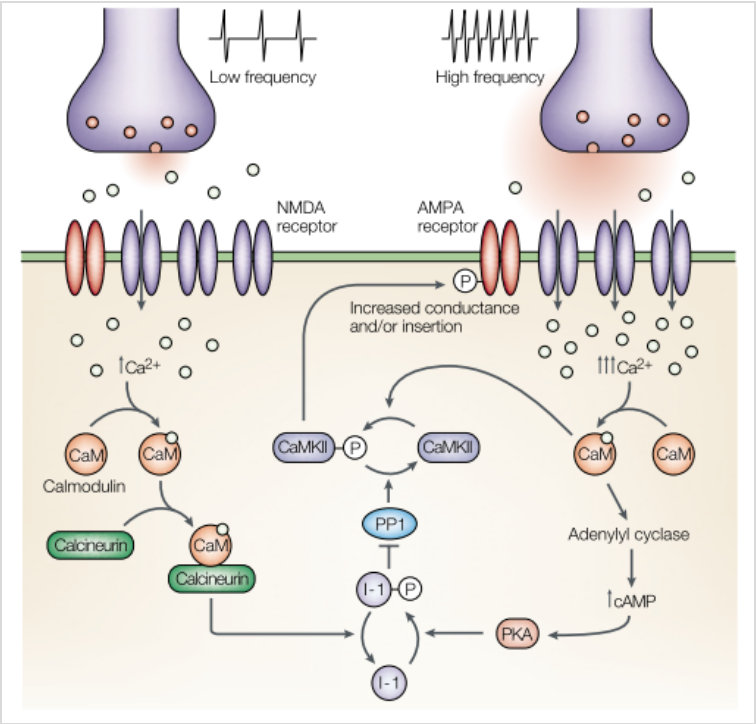


Figure 1.1. Schematic representation of NMDA receptors role in a persistent strengthening (LTP) and weakening (LTD) synaptic connection (Taken from Winder & Sweatt, 2001).

Neural plasticity and brain functioning

Neuroplasticity plays a crucial role in the development of the nervous system as it is wiring itself together also based on the experience is receiving during time; likewise, it plays a massive role in memory and learning, specifically in the hippocampus, the crucial brain region involved in storing memories and in learning process. LTD seems to be as important for learning as LTP (Ito & Kano, 1982; Martin, Grimwood, & Morris, 2000; Neves, Cooke, & Bliss, 2008; Sanes & Lichtman, 1999).

Modern neurosciences are investigating LTP and LTD relevant role in neuroplasticity attempting to exploit their potential mechanism to enhance brain functioning and

treat disorder and disease of the central nervous system (CNS) in a clinical perspective (Bliss & Cooke, 2011).

The structural changes tend to occur along with long-term plasticity even spontaneously, like in response to injury, including amputations. Emerging evidence from both human patients and animal models showed that, in some cases, such modifications might represent maladaptive plastic changes. The maladaptive plasticity refers to the plasticity in the nervous system that leads to a disruption of the function and may be considered as a disease state.

For instance, the phantom pain has been discussed to be related to plastic changes at several levels, especially involving the cortex (Flor, Nikolajsen, & Jensen, 2006); also functional neuroplastic changes along the sensory pathways, from the peripheral to central nervous system may contribute to the generation, development, and maintenance of neuropathic pain (Costigan, Scholz, & Woolf, 2009; Li et al., 2016; Woolf, 1989). Another mechanism that can be considered maladaptive could be referred to drugs effects, which can affect the LTP pathway causing a synaptic strengthening which can lead to addiction (Mameli & Lüscher, 2011; Van Den Oever, Spijker, & Smit, 2012).

In this manuscript, the main interest is investigating brain plasticity and how to enhance it starting from our exceptional capacity to learn, to acquire new skills or to restore impaired abilities which are undoubtedly one of the most investigated topics in cognitive neuroscience.

One way is the development of new protocols and the creation of innovative paradigms able to improve learning capacity and induce plastic brain changes.

Two studies in this thesis investigate the process of learning, both using a training paradigm. In the first study (see chapter 3), patients with amblyopia performed 8-days intensive perceptual training in combination with a non-invasive stimulation technique (discussed in the next paragraph) to improve contrast sensitivity and, based on the transfer of learning principle, also visual acuity (both impaired in amblyopia). A second study (see chapter 4), provided training lasting 8 sessions on cognitive functions, aimed to enhance information processing speed and inhibitory control in young healthy subjects. In this study, in one condition the training was associated with transcranial electrical stimulation with the goal of intensifying the benefits thanks to their combination. In this study we were interested in understanding if, from a generic cognitive training, performance in a specific task could have improved; in other words, if a general-to-specific cognitive transfer could have occured.

The scientific literature is full of studies on rehabilitative training paradigms resulting in improved performances: perceptual learning (Fahle, Poggio, & Poggio, 2002), cognitive training (Willis et al., 2006), and in particular working memory training, planning abilities, inhibitory control (Dahlin, Nyberg, Bäckman, & Neely, 2008; Jaeggi, Buschkuehl, Jonides, & Perrig, 2008; Li et al., 2008; Spierer, Chavan, & Manuel, 2013; Von Bastian & Oberauer, 2013) and modification of the activity of frontoparietal or medial frontal brain network (Beauchamp, Lee, Haxby, & Martin, 2003; Dahlin et al., 2008; Klingberg, 2010; Olesen, Westerberg, & Klingberg, 2004), and motor learning (Karni et al., 1998).

We are interested in any potential effect of the training on the improvement not only in the trained task but also in the transfer of learning to similar untrained tasks with possibly long-lasting learning effects (Schmidt & Bjork, 1992).

Although behavioural training are continually being advanced and progressed to adapt for each individuals' needs, the use of additional techniques known to induce neuroplasticity is on the rise. As mentioned before, not long past finding of a beneficial effect of expression of neural plasticity is the use of non-invasive brain stimulation (NIBS), precisely transcranial electrical (tES) and magnetic stimulation (TMS), of the cerebral cortex.

The following section will focus on such techniques, emphasising the ones known as Transcranial Electrical Stimulation (tES), with a specific focus on transcranial random noise stimulation.

## 1.2. Non-invasive brain stimulation techniques

The interest in the effects of non-invasive brain stimulation techniques (NIBS) when applied to human scalp began more than two centuries ago. In 1804, the Italian scientist Aldini reported the first successful treatment of patients suffering from melancholia using direct current application (Priori, 2003). Many other studies have been conducted; for instance, around 1902, an electrotherapy stimulation called Electrosleep was used with the intent of inducing a sleep-like state through (Robinovitch, 1914).

More recently, the evolution of NIBS over the past 30 years of research has led to a main differentiation between transcranial magnetic stimulation (TMS) and transcranial

electric stimulation (tES). By modulating cortical activity these techniques are both used in cognitive neuroscience as a method to investigate the relationship between cognitive processes and the functioning of the neural structures of the related brain area (Miniussi, Harris, & Ruzzoli, 2013).

Nowadays, NIBS techniques have become one of the favourite tools for neuroplasticity invastigation, as well as for diagnostic and therapeutic purposes.

In the next paragraph encompasses a description of TMS tool and more in details tES protocols, with a specific paragraph dedicated to transcranial random noise stimulation (tRNS), the main stimulation technique used in all the three studies of this manuscript.

## Transcranial Magnetic Stimulation

The first encouraging demonstration of transcranial magnetic stimulation (TMS) on humans has been performed by Baker in 1984 (Barker, Jalinous, & Freeston, 1985).

TMS operates on Faraday's principle of electromagnetic induction: an electrical current passing through one coil produces a magnetic field that in turn causes current to flow in a second conductor, which, in our case, is represented by the brain.

As shown in Figure 1.2, through the coil, a magnetic field is generated due to a set amount of current delivered by the TMS machine in a short period , which, is rapid enough to induces an electric field in the underlying cortex; the result is a modulation of e the resting membrane potentials or even an induction of neuronal firing (Figure1.2) (Barker, 1999; Jalinous, 1991).

TMS operates on Faraday's principle of electromagnetic induction: an electrical current passing through one coil produces a magnetic field that in turn causes current to flow in a second conductor, which, in our case, is represented by the brain.

TMS represents the most well-known method used to influence the excitability of the brain (Barker et al., 1985).

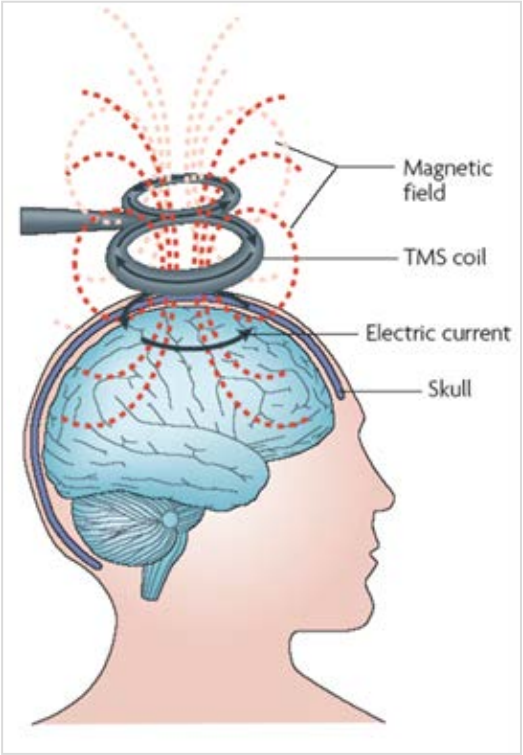


Figure 1.2. Schematic representation a TMS figure-eight coil inducing a magnetic field penetrating the scalp and skull which causes an electrical currents in the area of the brain beneath the coil (Taken from Ridding & Rothwell, 2007).

As shown in the image above, the coil represented, called figure-eight coil, consists of two adjacent wings allowing a focal stimulation of superficial cortical regions below the central segment of it. The neuronal fibres laying under the central segment of the coil within the region targeted are the most likely to be stimulated (Basser & Roth, 1991; Chen, Yung, & Li, 2003; Roth & Basser, 1990). The induced electrical stimulus activates

a mixture of local and far neurons due to the long axons projections to and from the site of stimulation, some excitatory, others inhibitory (Huerta & Volpe, 2009; H. Siebner & Rothwell, 2003).

Most of the knowledge about TMS effects on the human cortex comes from studies of the sensory cortex and even more, regarding the primary motor cortex (M1).

This relatively simple and painless TMS procedure, when applied on M1 can evoke a behavioural response, called motor evoked potential (MEP), which is a measurable physiological measure and it can be recorded using electromyography (EMG) (Figure 1.3).

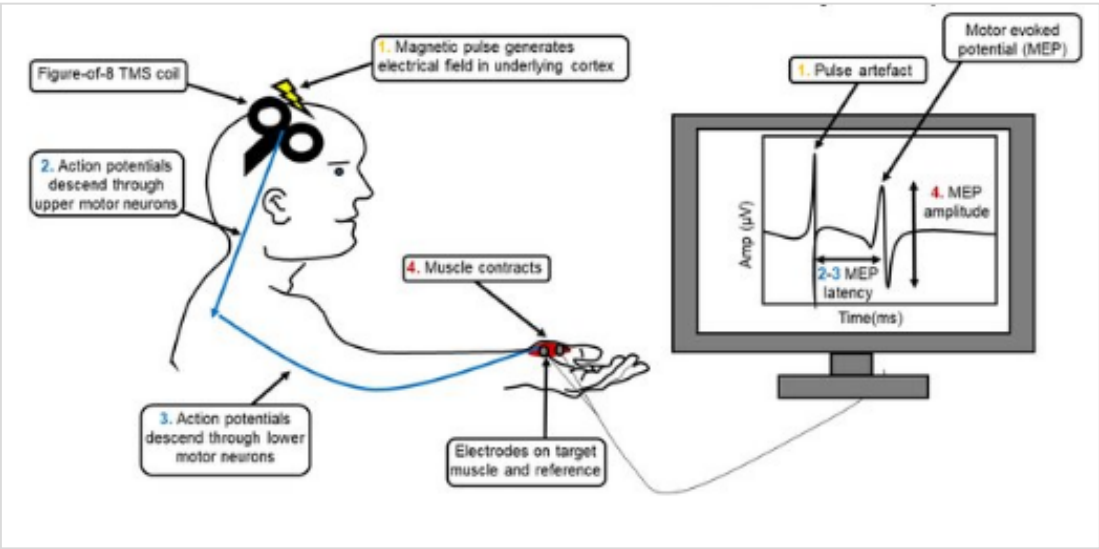


Figure 1.3. Schematic illustration of TMS-induced corticospinal activation registered through a pair of electrodes on contralateral FDI muscle; EMG signal showing a TMS-induced MEP (Taken from McMackin et al.,2019).

TMS pulse activates the corticospinal path predominantly via interneurons in the superficial cortical layers (Di Lazzaro et al., 2007); more specifically, epidural recordings show that TMS activates pyramidal tract neurons trans-synaptically (Di Lazzaro et al., 1998, 2006).

TMS application on the motor system can provide information about the excitability of the motor cortex, the conduction along corticospinal as well as the function of nerve roots and peripheral motor pathway to the muscles and moreover, the functional integrity of intra-cortical neuronal structures (Kobayashi & Pascual-Leone, 2003).

The investigation of the brain functioning has led to the development of different protocols, precisely: single pulse, paired-pulse and repetitive pulse (Parkin, Ekhtiari, & Walsh, 2015; Rossi et al., 2009), which represent excellent methods for measuring brain plasticity.

TMS protocols

The choice of using a specific stimulation protocol depends on the purpose for which TMS is used since each protocol induces a different effect on brain behaviour and functioning (Figure 1.4).

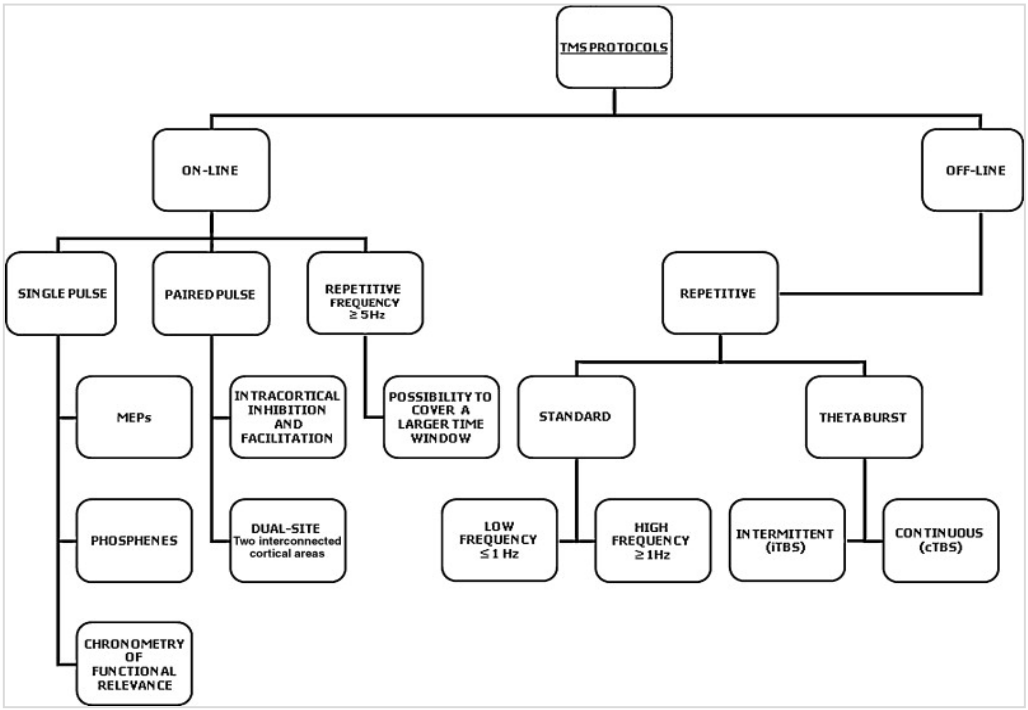

Figure 1.4. Schematic representation of different TMS protocols. (Adapted from Sandrini et al., 2011).

- Single-pulse TMS is applied to motor cortex (M1) to investigate the cortico-spinal excitability. By recording the motor threshold of M1 through motor evoked potentials (MEPs), is possible to obtain a measure of excitability of cortical neurons (Maeda, Keenan, Tormos, Topka, & Pascual-Leone, 2000).

- Paired-pulse TMS consists of a sub-threshold conditioning stimulus preceding a supra-threshold test stimulus at a given inter-stimulus interval (ISI). A short ISI, 1-5 milliseconds, seems to induce a short interval cortical inhibition (SICI) due to an interaction with GABAergic interneurons (Di Lazzaro et al., 2000, 2005), whilst an ISI range of 7-20 ms, is adopted as a measure of intra-cortical facilitation in which glutamatergic interneurons are involved (Kujirai et al., 1993); moreover, a longer ISI between 50 and 200 ms induces a long interval cortical inhibition (LICI) (Valls-Solé, Pascual-Leone, Wassermann, & Hallett, 1992), as a reflection of $GABA_B$ receptors involvement. The advantage is that it can be applied with a single coil on the same brain area, or with two coils in two distinct brain areas (paired associative stimulation or PAS), to promote phenomena of neuronal plasticity such as LTP and LTD (Valero-Cabré, Amengual, Stengel, Pascual-Leone, & Coubard, 2017)

- Repetitive TMS (rTMS) consists in the application of regularly repeated TMS pulses at low (<1 Hz) or high (≥ 1 Hz) frequency, which allows the modulation of the excitability of a cortical area that persists even after the duration of the stimulation.

- A recent protocol of intensive rTMS, known as theta burst stimulation (TBS) consists of a burst of 3 stimuli at high-frequency (50 Hz), which is repeated at intervals of 200 ms (i.e., 5 Hz). It includes continuous TBS (cTBS), intermittent TBS (iTBS), and intermediate TBS (imTBS). It has been shown that cTBS tends to depress excitability of

the primary motor cortex whereas iTBS has the opposite effect (Huang, Edwards, Rounis, Bhatia, & Rothwell, 2005).

These different effects may be explained through several mechanisms of actions such as via LTD and LTP processes at synaptic level (explained in the previous paragraph), as well as through change in the state of neural excitability (Iezzi et al., 2008) or they may be due to the stochastic resonance. More in details, according to the state dependency theory, the effect of the TMS on the performance would be the result of the interaction between the actual intensity of the stimulation and the initial state of excitability of the neurons involved in the stimulation; consequently, the inhibitory or facilitative effect of TMS would be related not only to the intensity of stimulation, but also to the state of neuronal activity prior to stimulation. On the other hand, the stochastic resonance theory (Stocks, 2000) hypothesises that the effect of TMS is associated with the introduction into the brain neural noise, and its intensity may facilitate or inhibit the performance.

Therefore, it is likely that the mechanism of action of the TMS is modulated by several variables, and that only the integration between these different theories may lead to a full understanding of its functioning.

Another type of stimulation, named transcranial electrical stimulation, has gained much interest in neuroscience as a method to understand how the brain works, and it is the topic of the next paragraph.

## Non-Invasive Transcranial Electrical Stimulation

Transcranial electrical stimulation (tES) is not a new concept to science: in 1755, Charles Le Roy stimulated with electricals signals the optic nerve and visual cortex in the attempt to treat blindness. More recently, in 1980, Merton and Morton showed that electrical stimulation over the occipital cortex of an intact skull resulted in phosphene perception (Merton & Morton, 1980). Subsequently, in the last 20 years, the family of transcranial electrical stimulation (tES) techniques have exponentially grown as tools to investigate neuroplasticity effects on cortical activity.

TES consists in the application of a weak electrical current through the scalp. The electrical current is applied usually via two or more electrodes, and the current is conducted between electrodes through skin and skull (Vöröslakos et al., 2018), and through the scalp reaching the brain tissue, where it can interact with the ongoing neuronal activity (Figure 1.5).

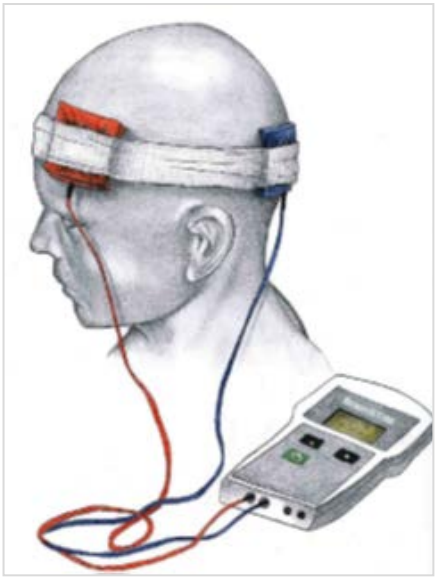

Figure 1.5. Representation of tES classic montage with a pair of electrodes positioned on left-DLPFC and occipital area V1 and connected to the stimulator.

Differently from the TMS, tES techniques are not powerful enough to elicit an action potential, but they modify the response threshold acting at subthreshold levels and changing the neuronal excitability (Radman, Ramos, Brumberg, & Bikson, 2009). By causing alterations of resting membrane potential, tES modifies neuronal synaptic efficiency (Liebetanz, Nitsche, Tergau, & Paulus, 2002; Nitsche et al., 2003).

TES include three main different protocols (Figure 1.6): transcranial direct current stimulation (tDCS), transcranial alternative current stimulation (tACS) and transcranial random noise stimulation (tRNS) (discussed in details the in next paragraph) (Fertonani & Miniussi, 2016; Paulus, 2011; Reed & Cohen Kadosh, 2018).

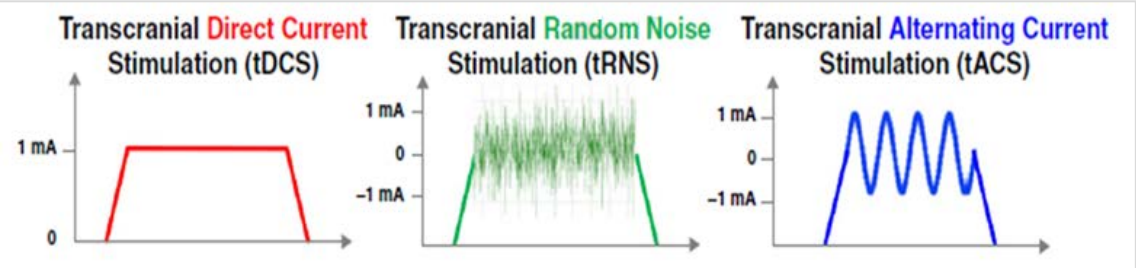


Figure 1.6. Examples of tES stimulation protocols. The vertical axis represents the current intensity in milliampere (mA), while the horizontal axis illustrates the time-course (Adapted from Santarnecchi et al., 2015).

## Transcranial direct current stimulation

tDCS consists in a direct current delivered through two electrodes (or a specific set of electrodes) named anode, the active one, and cathode, the reference or the return electrode. Many studies showed that direct current tES induces prolonged polarity-dependent cortical excitability alterations (Nitsche & Paulus, 2000), with an increasing in neural activity due to a facilitation of the anodal stimulation (a-tDCS) (Boros, Poreisz, Münchau, Paulus, & Nitsche, 2008) whereas a reduction or inhibition of neural activity

due to the cathodal stimulation (c-tDCS) (Ardolino, Bossi, Barbieri, & Priori, 2005). Both effects have been demonstrated by stimulating M1 and recording MEPs amplitude (Nitsche et al., 2008; Paulus, 2011).

Generally, stimulation durations ranging between 5 and 30 min, with a typical intensity range between 1 –2 mA (Bikson, Datta, & Elwassif, 2009).

Depending on several factors, such as, the montage, the intensity and the duration the effects of stimulation can persist beyond the end of the session; this effect is known as the aftereffect of stimulation (Nitsche & Paulus, 2011; Paulus, 2011).

The mechanisms of action of tDCS have been the most investigated among the electrical stimulation techniques. Some studies have shown that tDCS neuroplastic after-effects are NMDA receptor dependent: it has been observed a block of both short- and long-term effects of stimulation after post drug administration of NMDA receptor antagonist dextromethorphan (DMO). NMDA receptors have been reported to have a critical role in synaptic plasticity and LTP, affecting learning and memory processes (Liebetanz et al., 2002; Nitsche et al., 2003; Stagg & Nitsche, 2011). TDCS has also been associated to the increase of brain-derived neurotrophic factor (BDNF) which may alter synaptic plasticity in both animal and human cortex (Cheeran et al., 2008; Fritsch et al., 2010; Yoshii & Constantine-Paton, 2010). Increase of glutamate and glutamine concentrations is supposed to be involved in plasticity induction and related to alterations of intracellular calcium level (Hunter et al., 2015), similar to long-term glutamatergic potentiation and long-term depression in animal experiments discussed in the previous paragraph (Malenka & Bear, 2004).

Although many studies demonstrate that a-tDCS induce behavioural facilitation and c-tDCS yields the opposite effect via inhibition, as stated for TMS mechanism we cannot consider tDCS effects just as the results of facilitation or inhibition inductions but a consequence of the interaction between the stimulation parameters and the ongoing neural activity or brain state.

One distinct disadvantage of tDCS is that it may result in neurophysiological homeostasis of the populations of neurons stimulated (Siebner et al., 2004); that could result in an adjustment of the threshold of the system based on the constant input or a kind of return to their initial neural activity.

## Transcranial alternating current stimulation

TACS is the application of an electrical current that alternates between electrodes, usually in a sinusoidal wave applied with a specific frequency.

Differently from tDCS which alters neuronal excitability, tACS is said to interact with on-going rhythmic cortical activity during sensory or cognitive processes. TACS is polarity-independent, since it is an alternating current there is a continuous alternation between the cathode and anode electrodes. TACS generates the so-called entrainment, a synchronisation of the neuronal firing of a large number of neurons with the external induced frequency (Battleday, Muller, Clayton, & Cohen Kadosh, 2014). A causal effect between beta oscillation in the motor cortex and voluntary movement has been shown in many studies: the synchronisation of networks in the motor cortex to the 20 Hz frequency induced by tACS reduced the velocity of the execution of the movement (Pogosyan, Gaynor, Eusebio, & Brown, 2009), and

increased the duration of automatic inhibition (Cappon, D'ostilio, Garraux, Rothwell, & Bisiacchi, 2016); these studies contribute to potential clinical application in pathology related to motor system like Parkinson's disease.

The ability to selectively entrain neural oscillation seems to be more effective when tACS is applied at the same frequency as endogenous oscillations (Fröhlich & McCormick, 2010; Reato, Rahman, Bikson, & Parra, 2010); tACS may be useful to investigate the causal links between endogenous brain oscillations and specific cognitive processes.

Evidence showed that tACS effect is brain-state dependent. During a concurrent cognitive task, the modulation of activity induced by tACS was found during high demand (Alagapan et al., 2016; Violante et al., 2017).

Furthermore, it seems that this type of current could modulate the connectivity altering local $GABA_A$ levels (Stagg 2014) with an outlasting effect between the areas stimulated (Bächinger et al., 2017). Voltage-gated ion channels, specifically the activation of potassium and sodium channels, are thought to be the contributory cause of the aftereffect observed (Francis, Gluckman, & Schiff, 2003).

## Transcranial Random Noise Stimulation

TRNS has been investigated in the last decade, and it is the most recent tES method; the fact that is relatively new makes itself a fascinating exploration field.

TRNS, similar to tACS, consists in the application of an alternating current with the distinctiveness of randomly alternates within a range of frequencies. As tACS, it is polarity independent. Conventionally, the entire spectrum of frequency is from 0.1 Hz

to 640 Hz, and the current subdivisions include low frequency (lf-tRNS) from 0.1 Hz to 100 Hz and high frequency (hf-tRNS) from 101 Hz to 640 Hz (Fertonani, Pirulli, & Miniussi, 2011; Terney, Chaieb, Moliadze, Antal, & Paulus, 2008).

TRNS parameters

Several studies, manipulating each stimulation-factor (duration, intensity, montage, frequency band, timing) while controlling the other parameters, aimed to understand the relative contribution to explain the differences among the effects induced by the stimulation. A few studies demonstrated how tRNS differently modulate the cortical excitability depending on the frequency bands set. By measuring MEPs amplitude, Terney et al. (2008) showed that 10 minutes of hf-tRNS applied on M1 enhanced cortical excitability, with a long-lasting effect of an hour after stimulation. The neuromodulatory effect was selective for the high-frequency band when compared to the low frequency band. In the same study tRNS increased the behavioural performance, inducing a reduction in reaction times, suggesting early consolidation in implicit motor learning (Terney et al., 2008). Another study by Campana et al. (2016) showed that low- and high-frequency bands induced an opposite effect in a visual motion adaptation task (Campana, Camilleri, Moret, Ghin, & Pavan, 2016). Moreover, a study where the effect of hf-tRNS, lf-tRNS, a-tDCS, c-tDCS, and Sham stimulation were compared, revealed no significant difference between hf-tRNS and lf-tRNS, however hf-tRNS resulted having the most considerable effect on the participant's performance (Fertonani et al., 2011).

Regarding the stimulation duration a minimum of 4 minutes is required to obtain a significant modulation of cortical excitability, precisely, a transient reduction in BOLD response in sensorimotor cortex (Chaieb et al., 2009) and 5 minutes of tRNS induced a significant enhancement in MEP amplitude (Chaieb, Paulus, & Antal, 2011).

Concerning the intensity of the current delivered, the most common range used is between 1 and 2 mA. Moliadze and colleagues (2012), to verify the minimum amount of current required to induce an effect, compared tRNS and tACS to Sham at different current intensities (0.2, 0.4, 0.6, 0.8, 1 mA), stimulating for 10 minutes M1 in healthy subjects. The results demonstrated that tRNS set with an intensity current of 0.4 mA induced a reduction of MEP amplitude from 20 to 90 minutes after the stimulation; on the contrary, a higher intensity of 1 mA was able to increase the MEP amplitude immediately after the stimulation with a long-lasting effect of 90 minutes. A similar pattern was found for tACS stimulation (Moliadze, Atalay, Antal, & Paulus, 2012).

Additionally, the timing of application of the stimulation, before the task/training, (offline) or during (online), has been shown yielding to different facilitatory effects. From a study by Pirulli and colleagues (2013), tRNS resulted in improving more effectively the performance when applied online, so during the task execution. This finding supports the importance of stimulation timing (Pirulli et al., 2013).

A problematic issue using non-invasive electrical techniques is the cutaneous perception provoked by the delivery of the current; this side-effect is preferred to be avoided or at least reduced not only for the comfort of the participant but also to blind participants to the condition. A solution is the Sham condition which represents the control for active stimulation, and it consists of the delivery of the stimulation for just

30 seconds. This option contributes to maintaining similar blinding properties across real and active stimulation. However, tRNS, a-tDCS and c-TDCS, when compared in terms of cutaneous perception in three groups with naïve, experienced and investigators participants, showed a significant difference among conditions with a great advantage of tRNS which showed higher cutaneous perception threshold and lower response rates when compared with both tDCS (Ambrus, Paulus, & Antal, 2010).

The effects induced using tRNS has been explored in many fields and in comparison with other tES techniques. Most of the studies have been conducted on the motor cortex for the possibility of recording the MEPs which are considered a direct measure of the cortical excitability.

TRNS and motor cortex

After the pioneering study by Terney and colleagues (2008) who found that hf-tRNS increased the M1 excitability with a lasting effect up to 60 minutes, numerous other studies have been conducted by varying some parameters and comparing the technique with the other tES protocols.

In a study comparing a-tDCS, c-tDCS, tRNS and Sham stimulation for 10 minutes using 2 mA intensity over the leg motor cortex, both tRNS and a-tDCS induced an increase of the amplitude of the MEPs, with an immediate effect of tRNS with a duration of 40 minutes following the stimulation (Laczó, Antal, Rothkegel, & Paulus, 2014).

Moreover, 10 minutes of 1 mA tRNS on M1, compared to a-tDCS and tACS protocols resulted in the largest significant increase in cortical excitability and, compared to

Sham stimulation, tRNS significantly increased MEPs amplitude up to 20 minutes after stimulation (Inukai et al., 2016).

When 1 mA tRNS was compared with a-tDCS and i-TBS, the results confirmed a significant enhancement of the M1 excitability induced by all three types of active stimulations compared to Sham stimulation, with no significant difference among the types of active stimulations, although tRNS resulted in the strongest effect with an increase in excitability up to 60 minutes and a-tDCS in the longest MEP increase up to 90 minutes (Moliadze et al., 2014).

Furthermore, a combined study measured brain activity changes using functional magnetic resonance imaging (fMRI) method during the application of 1 mA of a-TDCS, lf-tRNS, hf-tRNS and Sham stimulation on M1 the first 10 minutes of a visuomotor learning paradigm. Hf-tRNS and c-tDCS showed a tendency to improve learning and lf-tRNS to worsen it; these effects remained for 20 minutes after stimulation in the late learning phase (Saiote et al., 2013).

TRNS and visual domain

Within the visual domain, in a study by Fertonani and colleagues (2011), different brain stimulation protocols specifically hf-tRNS, lf-tRNS, a-tDCS, c-tDCS, and Sham stimulation were applied to early visual areas to investigate their effects on the performance of an Orientation Discrimination Task (ODT) in one hundred and seven healthy participants. The findings revealed a distinctive consequence depending on the condition of the learning effect observed during the task execution and the resulting performance. Results showed that tRNS significantly improved performance accuracy

compared with a-tDCS, c-tDCS, Sham, and Cz stimulations (Fertonani et al., 2011). In conclusion, their results support the efficacy of hf-tRNS of the visual cortex over other stimulation protocols in improving behavioural performance on a visual discrimination task.

Other studies on clinical populations suggested that tRNS influenced the perceptual learning leading to a faster and more effective performance as well as transfer to untrained visual functions, such as visual acuity (VA) and contrast sensitivity (CS) in people with mild myopia and amblyopia (Camilleri, Pavan, Ghin, & Campana, 2014; Campana, Camilleri, Pavan, Veronese, & Giudice, 2014).

TRNS and cognition

Furthermore, moving to the cognition field, a recent study showed that three repeated sessions of active tRNS separated by 30 minutes produced an acute decrease in reaction time (RT) in the Go/NoGo task, precisely during the Go trials, suggesting the ability of tRNS in increasing the response, in other words the speed of execution; a long-lasting effect, when compared to Sham stimulation, was reported (Brevet-Aeby, Mondino, Poulet, & Brunelin, 2019).

Many other studies showed tRNS efficacy when associated with training. For instance, five days of tRNS applied on bilateral dorsolateral prefrontal cortex (DLPFC) coupled with cognitive training, induced long-term enhancement of cognition, specifically in calculation and memory recall learning, with a long-term modulation of neuron plasticity showed through near-infrared spectroscopy (Snowball et al., 2013). Moreover, tRNS has been demonstrated to boost the outcome of cognitive training,

precisely a numerosity discrimination task, result obtained when the training and the stimulation on the parietal lobes were associated, with a long-term effect up to 16 weeks (Cappelletti et al., 2013

Additionally, the possibility of a transfer effect has been investigated comparing four stimulation protocols tRNS, tDCS, multifocal tDCS and multifocal tACS with specific montages (F3-Fp2 for tDCS and F3-F4 for tRNS), combined with 9-sessions executive functions training. All the protocols, apart from the multifocal tACS, showed far transfer effects to fluid intelligence (Brem et al., 2018).

TRNS mechanisms of action

Regarding the mechanism of action of tRNS, it is still not clear how this random noise electrical application influences brain activity.

Currently, one of the most accredited theoretical models explains the effects of the tRNS (and, in general, of the NIBS) as a modulation of neural activity that modifies the relationship between signal and noise (Miniussi et al., 2013).

The brain represents a non-linear system in which the "signal" refers to the neural activity necessary for the processing of a target stimulus; the "noise" instead, describes the random neural background activity, constantly present in the system and which it does not seem to influence the coding of the target stimulus.

It has been shown that sensory information in the brain is represented through particular "neural coding" processes. One of the ways how this encoding takes place is related to the frequency rate or "rate coding". If the strength of a stimulus increases, a population of neurons responds by increasing its frequency rate and the number of

neurons involved. If the frequency and the number of neurons activated increase, the RTs and accuracy might be improved. On the contrary, the activation of a reduced number of neurons and a low discharge frequency (presence, therefore, of a weak signal), might determine slow RTs and low levels of accuracy in detecting a stimulus target. The final response of the system will be determined not only by the strength and intensity of the signal but will be based on the signal to noise ratio. Thus, the tRNS might act on the brain modifying this ratio. Since the tRNS is not able to stimulate focally, but it activates a large number of neurons, it could act as an introduction of noise. Thus, the final response given to a stimulus results from the interaction between the state of activation of the system, determined by the on-going processing and the noise added by the stimulation. The noise introduced with the stimulation might act both on the target neurons and the other neurons, changing the sensitivity of the system (Miniussi et al., 2013). However, this induced noise will not be a random element but will depend on the state and characteristics of the stimulated area. Therefore, tRNS could compromise or improve performance during a certain task, depending on the amount of noise introduced and the noise level already present in the system. In this regard, it is good to consider the concept of stochastic resonance (Figure 1.7).

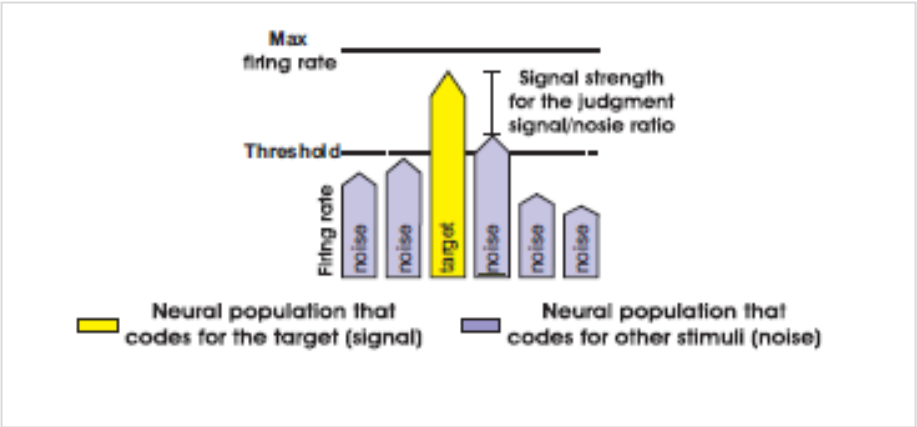


Figure 1.7. Illustration of the relation between the target signal (yellow) and other non-target signals (grey). The threshold represents the minimum intensity of a signal to reach the level to be included in the final subjective judgement (Adapted from Miniussi et al., 2013).

As shown by McDonnell & Abbott (2009), the presence of noise in a nonlinear system could be better for output signal quality than its absence. In this specific contest, the nonlinear system is the brain, and the goal is to find the right amount of noise to add in order to obtain the maximum efficiency of the system.

We refer to stochastic facilitation when random noise enhances the detection of weak stimuli and the information content of a signal (McDonnell & Ward, 2011; Moss, Ward, & Sannita, 2004).

In animal studies, short term (250ms) random noise stimulation (RNS) in vitro was shown being sodium channel-dependent (Remedios et al., 2019), and it induced inward sodium channel within the neurons caused by a faster reopening of these channels and a weak membrane depolarisation (Schoen & Fromherz, 2008).

Basing on the previous study, Chaieb and colleagues (2015) using a combination of active drugs, showed that carbamazepine (CBZ), a voltage-gated sodium channel blocker, significantly inhibited the excitability of the motor cortex enhanced by tRNS effect, lasting up to 60 minutes. They also reported that the administration of

dextromethorphan (NMDA receptor antagonist), did not block the tRNS-induced excitability increase (Chaieb, Antal, & Paulus, 2015). Indeed, tRNS seems to act increasing sodium channel activity (Terney et al., 2008), differently from tDCS, which instead has been demonstrated being NMDA receptor-dependent (Liebetanz et al., 2002). Moreover, in a different way from tDCS, tRNS has been suggested to induce repeated subthreshold stimulations, preventing homeostasis of the system.

Another potential mechanism of action could be due to the particular shape of tRNS, which for being a random and subthreshold stimulation, might cause temporal summation, via small membrane depolarisations, resulting in a neural activity enhancement by strengthening synaptic transmission efficiency, thus increasing performance (Fertonani et al., 2011).

In conclusion, the use of non-invasive electrical stimulation, specifically tRNS, to enhance the effect of a training is another recent finding of the beneficial effect of expression of neural plasticity (Fertonani et al., 2011; Moret et al., 2018; Santarnecchi et al., 2015).

Next chapters discuss practical applications of random noise stimulation in boosting neural plasticity in motor, visual and prefrontal cortex in the order listed.

# CHAPTER 2. TRANSCRANIAL RANDOM NOISE STIMULATION: THE FULL HIGH-FREQUENCY RANGE IS EXCLUSIVE FOR A CORTICAL ENHANCEMENT

## 2.1. Introduction

Neuroplasticity, as discussed in detail in the previous chapter, is a process that allows the brain to self-reorganise and to adapt continuously to new environmental situations (Moro et al., 2010).

In this study, we are interested in the possibility to influence cortical excitability and intervene on the plasticity of the nervous system through non-invasive cerebral neuromodulation methods. Here we will take into consideration cortical excitability of the primary motor cortex (M1).

Motor cortex excitability

M1 is located between the supplementary motor cortex (SMA) and the somatosensory cortex (Figure 2.1) and, in respect to the other brain regions, its excitability, thanks to the motor evoked potentials (MEPs) which constitute an electromyographic response of the muscles of interest, is easier to measure. MEPs are an extremely important and reliable measure to investigate cortical excitability and conductivity of corticospinal motor pathways (Rossini et al., 2015).

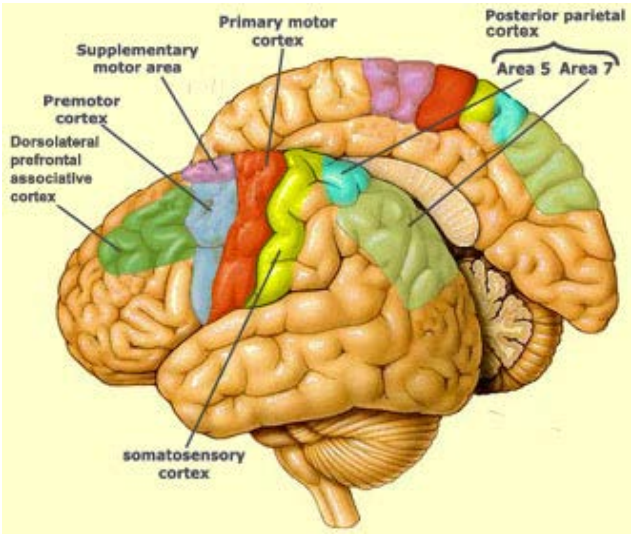


Figure 2.1. Anatomical representation of the primary motor cortex (M1) and other cortical areas involved in the motor system.

To date, TMS is one of the best tools for mapping brain functions and exploring cortical excitability (Rossini et al., 2015). Since its first application, it has been used in the neurophysiological field to investigate anomalies in the corticospinal tract and motor conduction processes (Ziemann, 2017) and, subsequently, with the introduction of repeated stimulation protocols, also in the neurorehabilitation field. TMS promotes brain plasticity and, potentially, the reorganization of damaged functional networks.

By applying TMS on M1, the motor interneurons are depolarised; this depolarisation is followed by the activation of the cortico-spinal tract ending with the generation of an involuntary contraction of the contralateral muscles, which can be recorded using an electromyograph. Thus, a MEP reflects the motor command sent from M1 to the motor neurons (Figure 2.2).

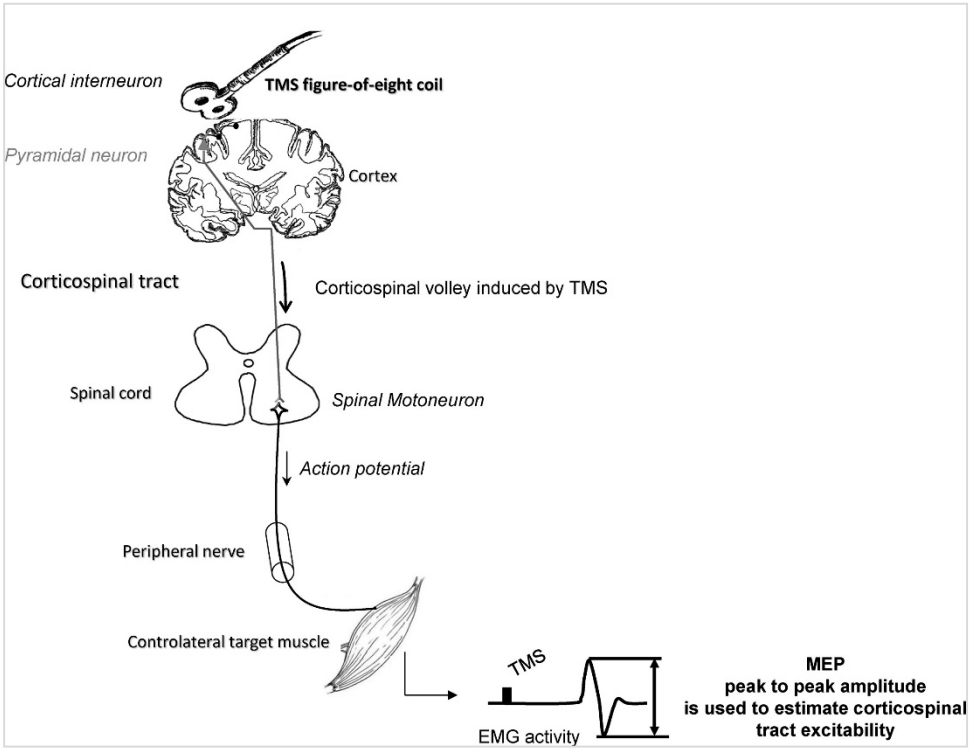


Figure 2.2. Simplified scheme of the mechanism of action of TMS on motor cortex (Adapted from Klomjai et al., 2015).

The MEP measurement provides lot of information: its size represented by the amplitude, usually expressed as peak to peak, its waveform, consisting of a start point (onset) and an endpoint (end), located on the same plane, with two deflections, positive and negative, named peak, and the latency, which corresponds to the interval between the stimulus and the beginning of the evoked potential (Figure2.3).

The MEP amplitude reflects the modulation of the excitability of the cortex through the cortical pathway, and the facilitation of neurons in the spinal cord or the brainstem.

The total latency time consists of a central delay (central delay), which is the time required for activation of the alpha spinal motor neurons and a peripheral delay, which represents the time between motoneuron activation and muscle response (Rothwell et al., 1999). It depends on the stimulated brain area, for example, in the hand muscles

latency is about 20 ms after stimulation, while it is shorter for the muscles of the face and longer for the leg's muscles.

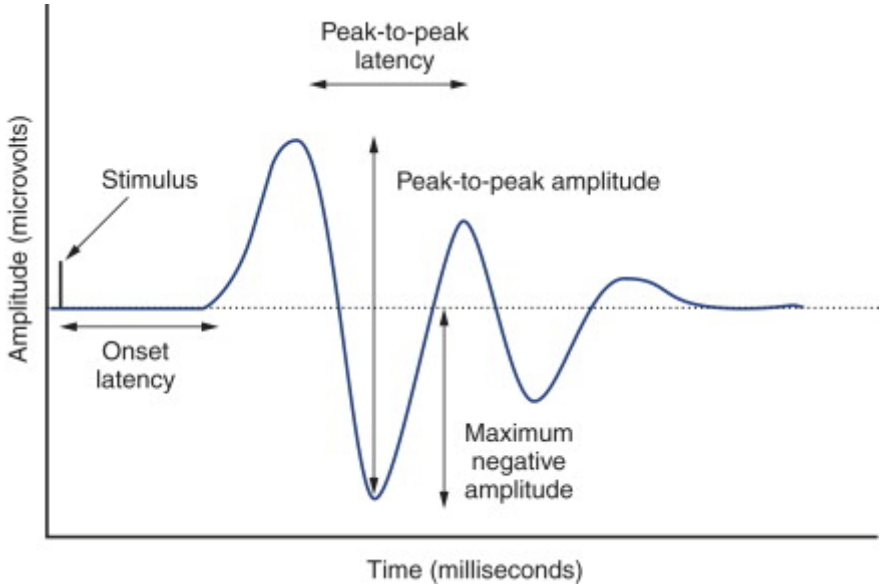

Figure 2.3. Example of an evoked potential response curve (Taken from Lieberman, 2008).

The cortical motor threshold (MT) is defined as the minimum stimulation intensity required to induce a reliable MEP in the target muscle. Since the lower thresholds are found for the muscles of the hands and forearms, MT is often measured in these muscles. MT can be measured by visible muscle contraction, defined as the minimum "twitch" of the muscle. Estimation of MT based on muscle twitch is easier to perform. However, it is discouraged as it is associated with inter-individual variability and it is difficult to quantify (Rossini et al., 2015). The most common method for measuring MT is based on electromyography (EMG). In this case, MT is defined as the lowest TMS intensity needed to produce a MEP, whose amplitude is between 50 and 150 microvolts (mV), in at least half out of the 10 stimuli delivered (Sacco, 2013). MT can

be measured when the target muscle is at rest, providing the resting motor threshold (RMT) controlled through the EMG activity or determined during a slight tonic contraction of the target muscle, the active motor threshold (AMT). Usually, AMT is lower than RMT of about 5% -20% of maximum stimulator output (MSO).

There are extrinsic factors (for example, 'conditioning' stimuli preceding a 'test' stimulus; see Chapter 1, TMS protocols section) and intrinsic factors (for example, mental activity) that can modify the amplitude of MEPs increasing its variability and, therefore, the measure of the cortical excitability. For instance, a voluntary contraction of the target muscle facilitates cortico-motor excitability causing a larger MEP amplitude; thus, an increase in MEP amplitude can occur without changes in TMS intensity. Furthermore, it is important to consider the intrinsic fluctuations of neuronal excitability at the cortical and spinal level, which make the MEP amplitude very variable even in an apparently resting state, with the complete relaxation of the target muscle. This "physiological noise" must be taken into account when, for example, the threshold is measured in resting conditions and the mean MEPs amplitude is used as an indicator of the state of cortical excitability. The variability explained by this physiological noise is particularly relevant, especially in TMS studies where the measurements of the MEPs amplitude must be repeated several times during the same experiment. A TMS study conducted with neuronavigation is advantageous to monitor the position of the coil on the target area of the cortex and to correct any displacement in the position or any variation in the angle of the coil with respect to skull surface during repeated measurements.

MEPs, therefore, represent a relevant measure for cortical excitability, brain plasticity investigation in particular, and they are crucial in the clinical setting as regards, for example, neurological diseases that compromise corticomotor conduction, such as Parkinson's disease (Rossini et al., 2015).

TRNS and brain plasticity

In this study we used single-pulse TMS protocol to perform repeated measurements of cortical excitability of the same motor area, before and after the application of another electrical stimulation technique discussed previously: tRNS.

We already know that tRNS, compared to the other tES methods, is the most recent electrical technique; therefore the exploration of its mechanisms of action in the clinical and experimental field is still somehow limited (Miniussi et al., 2013).

TRNS is a non-invasive electrical stimulation of the brain whereby a weak alternating current oscillating at random frequencies is delivered through the scalp using a pair of electrodes.

The frequency band of tRNS can encompass a full range, typically from 0.1 to 640 Hz, or can be delivered at low- or high-frequency (by convention, respectively ranging from 0.1 - 100 Hz and 101 - 640 Hz) (Terney et al., 2008).

In the last few years, its popularity has gradually increased, leading to the investigation of its potential benefits in motor, sensory and cognitive fields.

As previously discussed (see TRNS and motor cortex section, Chapter 1), for the first time Terney and colleagues (2008) applied 10 minutes of tRNS on M1, recording MEPs amplitude of the first dorsal interosseous dorsal muscle (FDI); they demonstrated modulatory effects sustained over time, up to one hour after stimulation. This

pioneering evidence has also been later confirmed by other studies, which showed that tRNS was able to increase motor cortex excitability with long-lasting effect (Laczó et al., 2014; Moliadze et al., 2014; Terney et al., 2008).

Studies on sensory or perceptual processing showed, for example, that hf-tRNS can improve visual detection or discrimination (Ghin, Pavan, Contillo, & Mather, 2018; Pavan et al., 2019; van der Groen & Wenderoth, 2016), can enhance the perception of facial identity (Romanska, Rezlescu, Susilo, Duchaine, & Banissy, 2015) and facial expression of emotions (Penton, Dixon, Evans, & Banissy, 2017; Yang & Banissy, 2017). Visual motion adaptation, on the other hand, has shown to be either attenuated or enhanced depending on the frequency band used (Campana, Camilleri, Moret, Ghin, & Pavan, 2016). Findings on cognitive abilities revealed that hf-tRNS is even able to enhance arithmetic skills and calculation (Pasqualotto, 2016; Popescu et al., 2016; Snowball et al., 2013). Regarding clinical application, hf-tRNS has been applied for reducing pain in multiple sclerosis (Palm et al., 2016) and for decreasing motor cortex excitability in Parkinson disease, (Stephani, Nitsche, Sommer, & Paulus, 2011) obtaining good results, as well as for reducing depressive symptoms (Chan et al., 2012) and improving negative symptoms in schizophrenia (Palm, Hasan, Keeser, Falkai, & Padberg, 2013). Moreover, both lf-tRNS and hf-tRNS have shown promising results in reducing tinnitus intensity and distress (Claes, Stamberger, Van de Heyning, De Ridder, & Vanneste, 2014; Joos, De Ridder, & Vanneste, 2015; Vanneste, Song, & De Ridder, 2013).

As explained in the previous chapter, hf-tRNS has shown to be a promising technique for boosting perceptual and motor learning (Camilleri, Pavan, & Campana, 2016;

Camilleri et al., 2014; Campana et al., 2014; Contemori, Trotter, Cottereau, & Maniglia, 2019; Fertonani, Pirulli, & Miniussi, 2011; Moret et al., 2018; van Koningsbruggen, Ficarella, Battelli, & Hickey, 2016; Van Wezel & Britten, 2006).

Despite the growth of studies probing the beneficial effects of tRNS, only a few have focused on the influence of the various stimulation parameters such as stimulation intensity, stimulation duration and frequency band.

Studies on sensory processing found that only intermediate stimulation intensities can increase visual detection or discrimination, suggesting that the perceptual enhancement is based on the phenomenon of stochastic resonance (Pavan et al., 2019; van der Groen & Wenderoth, 2016).

As for the effect of stimulation duration, a minimum of 5 minutes hf-tRNS over the motor cortex is required to obtain a significant increase in cortical excitability lasting the next 10 minutes (Chaieb et al., 2011).

Furthermore, only a few studies investigated the effect of the frequency band selected for the stimulation. In the visual domain, Campana and colleagues (Campana et al., 2016) found that, while hf-tRNS delivered bilaterally over visual areas V5/MT reduced the duration of motion adaptation, lf-tRNS increased it. In the motor domain, the effect of lf- vs hf-tRNS applied on the motor cortex was probed with motor evoked potentials (MEPs). Terney and colleagues (Terney et al., 2008) found a consistent excitability increase after 10 minutes of hf-tRNS (but no effect of lf-tRNS) lasting up to one hour, as measured through both physiological measures and behavioural tasks. This result was partially confirmed by Laczò and colleagues: after 10 minutes of hf-

tRNS over the motor cortex, they found an increase in excitability for the following 40 minutes after stimulation (Laczó et al., 2014).

These discrepancies highlighted the need to deepen the knowledge related to tRNS and, above all, to better understand the definite effect of each parameter in order to create a specific protocol aimed to obtain the most substantial stimulation benefit.

Besides an arbitrary subdivision of the frequency spectrum into two frequency bands, lf-tRNS (0.1 Hz -100 Hz), and hf-tRNS (101 - 640 Hz), the effect of other frequency ranges on cortical excitability is still unknown. In particular, whether it is well established that the whole hf-tRNS band is able to produce an increase in cortical excitability (Inukai et al., 2016; Laczó et al., 2014; Moliadze, Fritzsche, & Antal, 2014; Terney et al., 2008), it is not clear if the whole frequency band used in hf-tRNS is necessary for inducing such a change, or whether sub-ranges of the high-frequency band are sufficient to provide a reliable effect.

To assess this hypothesis, we created two experiments. Experiment 1 compared the effects of two sub-ranges of the high-frequency band: the first spanning 100 - 400 Hz and the second from 400 to 700 Hz concerning Sham stimulation. It is indeed possible that these two sub-ranges of frequency might modulate the brain activity differently. Experiment 2 tested the effect of the whole high-frequency band from 100 to 700 Hz compared to Sham.

No study has ever directly explored possible differential effects of hf-tRNS by considering both the spectra and the width of the frequency bands.

To investigate and compare the modulatory effects of these different frequency bands, we measured MEPs amplitude variations of the FDI induced by single-pulse TMS.

## 2.2. Methods

Participants

A total of 14 healthy young adult female (mean age 21, range 19-25 years) of the University of Padova, took part in this study. More specifically, 8 out of 14 participants took part in both experiments, and 3 out of 14 participated in Experiment 1 or 2 only, so to obtain two groups of 11 participants each. All the participants had no TMS contraindications (Rossi et al., 2009) assessed through a written questionnaire, and they gave written informed consent according to the Declaration of Helsinki. All participants were right-handed (assessed by the Edinburgh Handedness Inventory) (Oldfield, 1971). This study was approved by the local Ethics Committee (Protocol Number: 2459).

One limitation of this study is that only female participants have been included. However, it is reasonable to assume that these results can be generalised to male participants or gender-balanced samples. In fact, a prolonged aftereffect of tDCS in females with respect to males was found, but only in terms of a reduced excitability due to cathodal stimulation, whereas no differences were found with the increased excitability due to anodal stimulation (Kuo, Paulus, & Nitsche, 2006). Since both anodal tDCS and hf-tRNS produce an increase of cortical excitability, it is reasonable to assume that ovarian hormones do not alter the effect of transcranial electrical stimulation in either case.

Apparatus

*Electromyography (EMG)*

Corticospinal excitability was assessed by measuring the amplitude of MEPs of the FDI by single-pulse TMS over M1 using a Magstim Rapid$^2$ stimulator.

The stimulator was wired to a computer where a Matlab script triggered 25 pulses with an inter-stimulus interval (ISI) of 10 seconds, delivered through a 70 mm figure-of-eight coil.

Surface electromyogram was recorded from FDI muscle of the right hand via Ag/Agl electrodes (the active electrode on FDI, the inactive one on the third phalanx of the index oh the same hand and the ground on the upper side of the wrist) in a belly-tendon montage. Using System PLUS Evolution software (Myohandy Matrix Line, Micromed) raw signals were amplified and digitised, setting a sampling rate of 2048 Hz and a bandpass filter of 5 - 600 Hz. The electrode impedance was kept below 10kΩ. The epoch considered was 200 ms and a time window between 5 and 50 ms was recorded after the TMS pulse to obtain the difference between the maximum and the minimum peak, automatically detected by the software.

*Stereotaxy and motor threshold (MT)*

For each participant, we first found the point of the skull closest to the Talairach coordinates of the hand area (Mylius et al., 2013; Niyazov, Butler, Kadah, Epstein, & Hu, 2005) using a frameless neuronavigation system (BrainSight 2.3.8 together with an NDI Polaris Vicra camera). Then, a 3 by 3 grid centred on the previously found site and with 1 cm distance between them was marked on the skull of each participant with

small stickers. Each point of the grid was tested with single-pulse TMS starting from an intensity of 30% of the maximum stimulator output (MSO) and increasing it in steps of 5-10% until MEPs equal or above 1 mV were elicited. The stimulation site of the right FDI was identified as the point eliciting the largest MEP, keeping the same TMS intensity. Once the final stimulation site was found, its coordinates were recorded and maintained equal for each participant during all sessions, using the stereotactic frameless neuronavigation system (Gugino et al., 2001). The RMT was defined at each session as the intensity of TMS needed to evoke ~1 mV peak-to-peak MEP amplitude. It was assessed with single-pulse TMS by increasing or decreasing TMS intensity (1-2%) till reaching the target of ~1 mV peak-to-peak MEPs mean amplitude and verified with successive 10 pulses with 4 s of ISI.

*Transcranial random noise stimulation*

The current was delivered by a battery-driven stimulator (BrainStim, EMS) using a pair of rubber electrodes covered by sponges soaked in saline solution. The target electrode was 16 $cm^2$ large, was positioned above the primary motor area (M1) and its centre matched the cortical representation of the FDI. The reference electrode, 60 $cm^2$ large, was placed above the contralateral orbitofrontal area. This position is widely used for positioning the reference electrode (Chaieb et al., 2015; Terney et al., 2008).

In all conditions but Sham, tRNS was delivered for 10 minutes with a current intensity of 1.5 mA and 0 mA offset. Current linearly increased in intensity up to 1.5 mA during the first 30 s of stimulation. In the Sham condition, the current linearly increased for the first 15 s up to a 1.5 mA and then decreased to 0 mA in the next 15 s. The current

density was maintained within the safety limits (i.e., below 1.0 A/$m^2$) (Poreisz, Boros, Antal, & Paulus, 2007).

Experimental procedure

Two different experiments have been run in this study (Figure 2.4).

Participants were seated in a comfortable chair with a mounted headrest throughout the experiments. Participants were blind towards the experimental conditions and were not able to distinguish between real and Sham stimulation.

25 MEPs using single-pulse TMS were recorded at baseline (immediately before stimulation) and after tRNS at 0, 10, 20, 30, 45 and 60 minutes after 10 minutes of tRNS stimulation. The ISI was set as 10 seconds to reduce any potential interference affecting MEP amplitudes due to consecutive TMS pulses with shorter ISI (Julkunen, Säisänen, Hukkanen, Danner, & Könönen, 2012). The coil was positioned around 45-degree rotation about the parasagittal plane to induce a posterior-to-anterior current in the underlying cortex.

The order of the stimulation conditions was counterbalanced within participants, with at least 2 days between sessions.

Experiment 1 consisted in two active tRNS sessions, Low-hf-tRNS (L-hf-tRNS) ranging from 100 Hz to 400 Hz and High-hf-tRNS (H-hf-tRNS) from 400 Hz to 700 Hz, and Sham stimulation.

Experiment 2 consisted in the Whole-hf-tRNS (W-hf-tRNS) from 100 Hz to 700 Hz and the Sham stimulation.

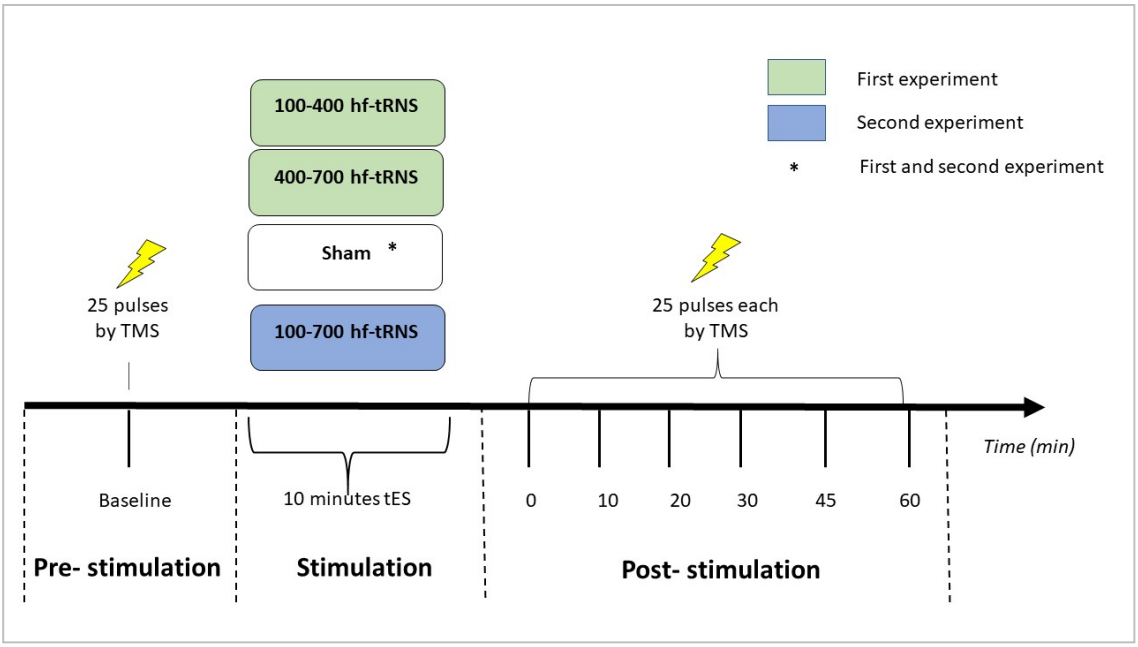


Figure 2.4. Experimental design representing the four stimulation conditions. 25 TMS-induced MEPs were recorded before tRNS (or Sham stimulation) and at each post-stimulation session.

Statistical analysis

System PLUS Evolution software (Myohandy Matrix Line, Micromed) automatically calculated MEP amplitude. We compared for each experiment, MEP amplitudes of each Stimulation condition at baseline (before stimulation): with a one-way repeated measures ANOVA in Experiment 1, and with a paired t-test in Experiment 2. Since no significant differences were found between any of the Stimulation conditions at baseline, all MEP amplitudes were standardised using the mean and standard deviation of the baseline of each session.

In order to have an overview of the data, we combined results of Experiment 1 and 2, and we run a mixed effect regression comparing a set of nested mixed-effects models (Pinheiro & Bates, 2000) with Stimulation condition (tRNS: Low-hf-tRNS, High-hf-tRNS, Whole-hf-tRNS, Sham) and Time (baseline, 0, 10, 20, 30, 45, 60 minutes post-stimulation) as fixed effects, with Participant, nested in Stimulation condition and Time

as random effects. A stepwise ANOVA for model selection (lowest AIC value and p-value) was used to identify the combinations of variables that best predicted the outcome variabilities. An effects plot (Fox, 2015) of the winner model was implemented.

We then separately analysed the results of the two experiments.

For Experiment 1, we applied Type III Analysis of Variance with a linear mixed-effects model and Satterthwaite's approximation of degrees of freedom (Kuznetsova, Brockhoff, & Christensen, 2017). Fixed effects were Stimulation condition (tRNS: Low-hf-tRNS, High-hf-tRNS, Sham) and Time (baseline, 0, 10, 20, 30, 45, 60 minutes post-stimulation]; random effects were Participant nested in Stimulation condition and Time. Likewise, for Experiment 2, we applied Type III Analysis of Variance with a linear mixed-effects model and Satterthwaite's approximation of degrees of freedom with Stimulation condition (Whole-hf-tRNS, Sham) and Time (baseline, 0, 10, 20, 30, 45, 60 min post-stimulation) as fixed effects and Participant nested in Stimulation condition and Time as random effects. Student's t-test was used to compare MEPs in a post-hoc analysis. Effects were considered significant with $p<0.05$.

## 2.3. Results

All participants well tolerated the stimulation protocol without reporting any side-effect.

In Experiment 1, a one-way repeated measures ANOVA showed no significant differences between Stimulation conditions (Low-hf-tRNS, High-hf-tRNS, Sham) at baseline ($F_{2,10}=0.74$, $p=.49$), and in Experiment 2, a paired t-test showed no significant

differences between Stimulation conditions (Whole-hf-tRNS, Sham) at baseline ($t_{10}$=1.16, p=.27). This implies that any differences between conditions arising from hf-tRNS could not be attributed to differences at baseline.

Table 2.1 shows the results of a mixed-effects model selection on the data set of the two experiments. Only the quadratic effect of Time and the effect of Stimulation condition (Low-hf-tRNS, High-hf-tRNS, Whole-hf-tRNS, Sham) in interaction with the quadratic effect of Time significantly increased the prediction capacity, the latter being the winner model.

| **Fixed effects** | **Model df** | **AIC** | **BIC** | **Chisq** | **Chi Df** | **Pr(>Chisq)** |
|---|---|---|---|---|---|---|
| | 4 | 27164 | 27192 | | | |
| **Time** | 5 | 27163 | 27198 | 3.7131 | 1 | 0.054 |
| **Time$^2$** | 6 | 27158 | 27200 | 6.1695 | 1 | 0.013 * |
| **Time$^2$ + Stimulation** | 9 | 27161 | 27224 | 3.6572 | 3 | 0.301 |
| **Time$^2$ * Stimulation** | 15 | 27140 | 27245 | 32.2726 | 6 | 1.447e-05 *** |

Table 2.1. The result of model comparisons in a set of mixed-effects models on merged data set of the two experiments. Df = degrees of freedom; AIC = Akaike's information criterion; BIC = Bayesian information criterion; Chisq = chi-squared statistic; Chi Df = chi-squared degree of freedom; Pr(>Chisq) = probability value; participants are random effects in each model.

More specifically, the amplitude of MEPs as a function of Time after tRNS can be described by an inverted U, but not for all Stimulation conditions. The Sham condition does not show any quadratic trend but just a slight linear increase, whereas both Low-hf-tRNS (100 Hz to 400 Hz) and High-hf-tRNS (400 Hz to 700 Hz) have a very mild curvature, compatible with a feeble modulation of cortical excitability.

Finally, Whole-hf-tRNS (100 Hz to 700 Hz) is the condition where the quadratic trend is more evident, and the after-effects are more consistent and persistent, as also shown in Figure 2.5.

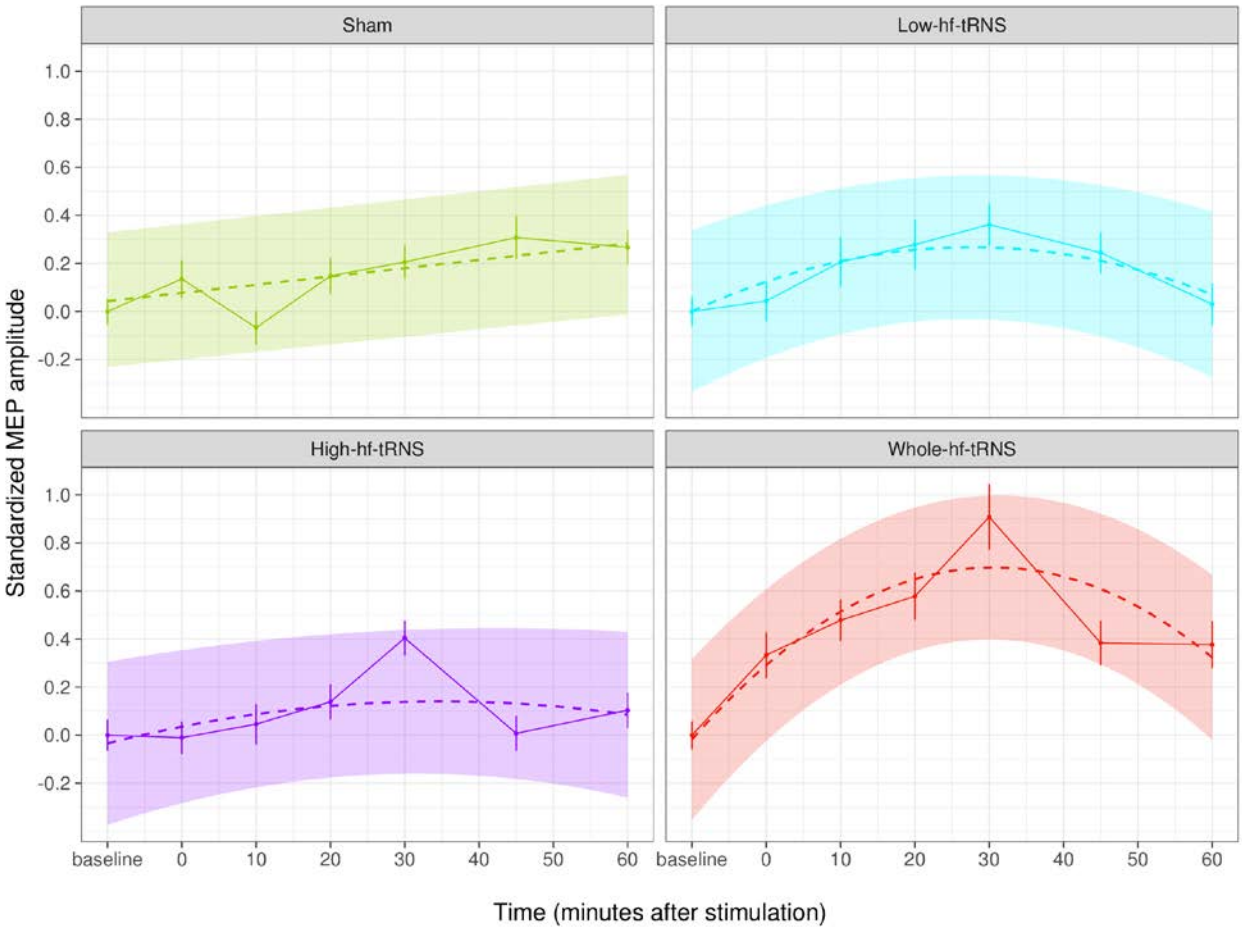


Figure 2.5. Effects plot for the predictors of the winner model (Time2 * Stimulation). Error bars represent standard error; coloured areas represents confidence bands.

In order to pinpoint the differences between the conditions more in detail, data of the two experiments were also analysed separately.

In Experiment 1, after Low-hf-tRNS (100 Hz to 400 Hz) or High-hf-tRNS (400 Hz to 700 Hz), a moderate and uneven increase in excitability is observable (Figure 2.6, left graph). However, the ANOVA applied to the linear mixed-effects model did not reach statistical significance either for the main effect of Stimulation condition ($F_{2,28.1}$=0.14,

p=0.86) or for the main effect of Time ($F_{6,59.9}$=1.22, p=0.30). The interaction between Stimulation condition and Time indeed reached statistical significance ($F_{12,5487.4}$=2.34, p=0.005), but the twelve post-hoc comparisons between each level of tRNS and Sham at 0, 10, 20, 30, 45, 60 minutes were not significant.

In Experiment 2, the Whole-hf-tRNS (100 Hz to 700 Hz) showed a considerable increase in excitability (Figure 2.6, right graph) instead. The two main effects were not significant (Stimulation condition: $F_{1,17.4}$=2.91, p=0.10; Time: $F_{6,60.8}$=1.72, p=0.13), but the interaction between Stimulation condition and Time reached statistical significance ($F_{6,3683.9}$=4.63, p<0.001). According to the post-hoc analysis, differences between tRNS and Sham condition were significant only at 10 ($t_{10}$=2.47, p=.032) and 20 minutes ($t_{10}$=3.06, p=.011) after stimulation.

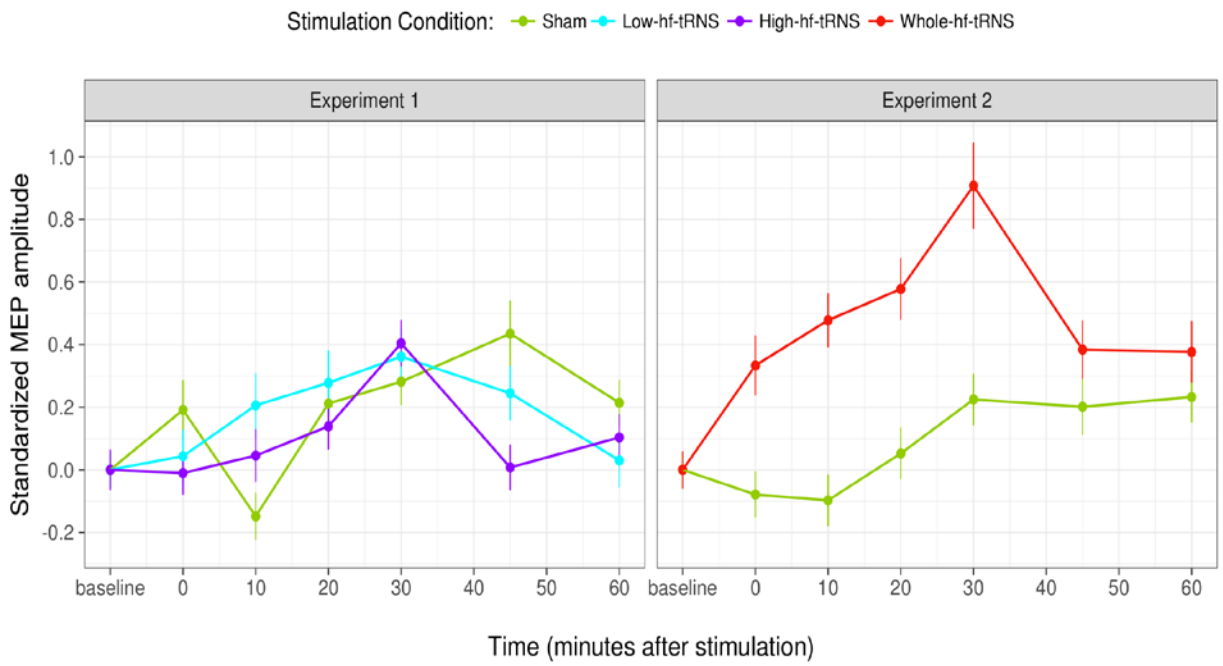


Figure 2.6. Results of Experiment 1 and 2. Standardised MEP amplitudes for different stimulation conditions at different time intervals from tRNS (or Sham stimulation); error bars represent standard error.

Considering all the results shown, while stimulating with the whole frequency band produced a relevant increase in neural excitability, with an effect lasting up to twenty minutes after stimulation, splitting the frequency range by half, and irrespectively of the specific (low or high) sub-range, strictly reduced the effects of the stimulation.

## 2.4. Discussion

In the last few years, tRNS has aroused considerable interest, especially for its ability to modulate cortical excitability compared to other tES techniques. With the present study, we intended to explore possible differential modulatory effects of different frequency ranges within high-frequency band, which so far, has shown to be the most promising in increasing cortical excitability and inducing cortical plasticity with a lasting effect up to an hour. Specifically, we aimed to better understand whether the increase in excitability of M1 due to hf-tRNS was mainly due to the lower, from 100 to 400 Hz, or, the higher part, from 400 to 700 Hz, of the high-frequency band (100 - 700Hz). In Experiment 1 we compared the effect of these two sub-ranges of frequency to Sham stimulation, and in Experiment 2, we tried to replicate (except few differences in the parameters used) the results of Terney and colleagues (2008) delivering the whole high-frequency band (100 - 700 Hz) and comparing the effects with those obtained with Sham stimulation.

Unexpectedly, the results of Experiment 1 indicated that Low-hf-tRNS or High-hf-tRNS produced only a very mild modulation of cortical excitability. This variation was captured both by a quadratic effect of Stimulation condition in interaction with the quadratic effect of Time in the model comparison with the combined data of the two

experiments, and by a significant interaction between Time and Stimulation condition with data of Experiment 1, although no significant differences were found in post-hoc t-tests between any of the two tRNS conditions and Sham stimulation.

In Experiment 2, instead, Whole-hf-tRNS (Figure 2.6, right panel) induced a much more noticeable modulation of cortical excitability. This visible inflexion was confirmed both by the interaction between Stimulation condition and the quadratic effect of Time in the model comparison with the combined data of the two experiments and by the significant interaction between Time and Stimulation condition. Here, post-hoc t-tests revealed a significant difference between Whole-hf-tRNS and Sham at 10 and 20 minutes after stimulation.

Thus, looking at the overall results, we obtained a very little effect (if any) on cortical excitability by splitting the high-frequency band of tRNS into two halves. Neither the lower half nor, the higher half of the high-frequency band seemed able to have a significant impact on cortical excitability. We supposed that by reducing the range of frequencies, we also reduce the amount of noise (e.g. maximally shrinking the frequency range we are left with a single frequency, removing all the noise) that might reduce the effect of tRNS on cortical excitability.

An important mechanism hypothesised on the physiological effects of tRNS is associated with the repetitive opening of sodium (Na+) channels via high-frequency stimulation (Schoen & Fromherz, 2008). Specifically, the tRNS acts by depolarising the neuronal membrane and determining the opening of the Na+ channels and since the entry of Na+ ions is insufficient, no action potentialis generated, but only a "local response". The repolarisation of the membrane occurs passively for a more extended

period than the duration of the entry of Na+ ions. Then, with repeated stimulation, the Na+ channels can reopen and induce a second flow of Na+ ions, which further depolarises the membrane, increasing the effect of the previous depolarisation. This leads to the development of mechanisms similar to LPT (see Chapter 1, Long-term plasticity section) (Fertonani & Miniussi, 2017; Schoen & Fromherz, 2008; Terney et al., 2008). Furthermore, the neuronal membrane is composed of numerous voltage-dependent ion channels and is subject to simultaneous flows of currents (Ca++ K+, Cl-) and tRNS can amplify the changes induced in the membrane fluctuation and, therefore, intensified effects can be obtained (Terney et al., 2008).

We know that the optimal modulation occurs for intermediate levels of intensity (Pavan et al., 2019; Remedios et al., 2019; van der Groen & Wenderoth, 2016), but it is not clear what is the optimal amount of noise in terms of the frequency range, except for the fact that high-frequencies are needed (Terney et al., 2008). Here, we demonstrated that reducing the usual high-frequency range, and thus reducing the noise, strongly impairs the modulatory effect of hf-tRNS on cortical excitability. It is possible that a lower amount of noise is not able to produce the same modulation of opening and closing of $Na^+$ channels.

Similar to previous studies (Laczó et al., 2014; Moliadze et al., 2014; Terney et al., 2008), hf-tRNS has been able to enhance MEP amplitudes in post-stimulation measurements. However, unlike these studies, here we have been able to reliably increase cortical excitability only up to 20 minutes after stimulation. Compared to the 60 minutes found by both Terney and Moliadze and colleagues (Moliadze et al., 2014; Terney et al., 2008), or the 40 minutes found by Laczó and colleagues (Laczó et al.,

2014), our modulation of cortical excitability was shown to be shorter, but comparable to what obtained by Inukai and colleagues (2016), (although they recorded MEP amplitudes only up to 20 minutes after stimulation).

Differences in the duration of modulation of cortical excitability might depend on many parameters of the electrical stimulation such as current type, current intensity, duration of stimulation, stimulation site, frequency range (for tRNS).

The main differences between the present and previous studies are stimulation intensity and ISI. Here, an ISI of 10 seconds has been used, instead of 4 seconds as in other studies (Terney et al., 2008). With this frequency of TMS pulses, even after Sham stimulation cortical excitability seemed to have a slight, although non-significant, increase across successive blocks (Figure 2, first panel). However, even if there was such a linear increase, this is unlikely to interact with the effect (if any) of tRNS. For what concerns stimulation intensity, in this study, we used 1.5 mA that might exceed the optimal intensity for modulating cortical excitability in terms of a persistent enhancement. Both Terney and Moliadze and colleagues (Moliadze et al., 2014; Terney et al., 2008) have successfully used 1 mA hf-tRNS in order to modulate MEP amplitudes up to 60 min; studies on perceptual mechanisms have found an optimal enhancement of performance with 1 mA, while further increasing the intensity of stimulation worsened performance (Pavan et al., 2019; van der Groen & Wenderoth, 2016).

On the other hand, using an even higher intensity (2 mA) of hf-tRNS, as Laczó and colleagues (2014), induced a long-lasting increase in cortical excitability up to 40 minutes, approximately lasting twice concerning Whole-hf-tRNS we obtained, with a quadratic trend similar to that found in the present study. However, Laczó et al. (2014)

used different parameters: first, the fact that tRNS was applied over a different cortical site. It is well known that the distance between the cortical surface and the skull varies greatly depending on skull position. Since the site stimulated was much closer to the sagittal midline where there is a more considerable distance between the skull and the cortex, it is likely that more of the current was diffused into the cerebrospinal fluid (which has higher electrical conductivity) and presumably less current arrived at the target location. Second, the size of the electrodes they used was more than double the size used by Terney et al. (2008) and in the present study. Both factors have likely decreased the amount of current reaching the target area, thus compensating the high intensity used in that study.

Moreover, we found a larger intra-variability in MEPs amplitudes. This underlines the importance of considering the intrinsic fluctuations of neuronal excitability (cortical and spinal) and the strong dependence of MEP amplitudes on the state of corticomotor excitability (Rossini et al., 2015). In our study, due to the longer ISI set, we delivered 25 pulses each recording, less than other studies in which 40 pulses were recorded. Analysing more MEPS might have contributed to MEP variability reduction. It is well-known that the effects of stimulation techniques, tRNS in particular, are, in fact, strongly dependent on the state of the brain system (Miniussi et al., 2013). Recent studies with concurrent TMS and electroencephalogram (EEG) showed that the amplitudes of MEPs depend on EEG phase and oscillations: these may explain, at least partially, the intra-trial variability of the MEPs amplitudes (Rossini et al., 2015) and suggests the importance of combining more techniques as in TMS-EEG studies.

Beside moderate intensities as suggested by other studies (Pavan et al., 2019; van der Groen & Wenderoth, 2016), also larger frequency ranges seem to yield a more pronounced effect in terms of increased excitability. However, these results should be carefully taken into consideration when tRNS is used in protocols aiming to improve brain functions and in light of the fundamental role of the brain state a future direction is to implement the experimental design with the EEG technique with a larger gender-balanced sample.

In conclusion, we suggest that an intermediate intensity of tRNS is optimal in increasing cortical excitability for a prolonged interval, whereas higher intensities can reduce this effect. The novel finding is that a large amount of noise (i.e. a wide range of frequencies) is needed to produce a significant and persistent increase in cortical excitability, while a smaller amount of noise (i.e. a narrower frequency range) seems not to induce such a modulatory effect.

## 2.5. Acknowledgements

A version of this chapter has been published in the Journal: Scientific Reports and has been accordingly cited in the reference section: Moret, B., Donato, R., Nucci, M., Cona, G., Campana, G. (2019). Transcranial random noise stimulation (tRNS): a wide range of frequencies is needed for increasing cortical excitability. *Scientific Reports,* 9(1), 15150.

# CHAPTER 3. TRANSCRANIAL RANDOM NOISE STIMULATION AND PERCEPTUAL LEARNING IMPROVE VISUAL FUNCTIONS IN ADULTS WITH AMBLIOPIA

## 3.1. Introduction

There is growing evidence that visual plasticity occurs not only during childhood as previously thought but also during all life in response to changes in sensory experience (Nelson, 2000).

When normal visual development is disrupted, idle or unexploited connections could be permanently pruned (Maurer, Lewis, & Mondloch, 2005) causing visual disorders that might persist all life, such as amblyopia, the topic of interest of this study.

Amblyopia

Amblyopia is a visual disorder due to an abnormal pattern of functional connectivity of the visual cortex and characterized by several visual deficits of spatial vision. It consists in a reduction of visual functions in one, or both eyes, regardless of the optimum optical correction and the absence of any pathology of the visual system (Ciuffreda, Levi, & Selenow, 1991).

It is a developmental disorder caused by reduced visual stimulation during early life (critical period), thus a consequence of that abnormal visual experience (Levi & Li, 2009).

Three are the most common causes of amblyopia: strabismus, which consists of a misalignment of the eyes, uncorrected anisometropia, which is an unequal refractive

error between the two eyes, or both strabismus and anisometropia (Giaschi, Chapman, Meier, Narasimhan, & Regan, 2015).

Amblyopia encompasses several spatial vision abnormalities such as reductions in visual acuity (VA) and contrast sensitivity function (CSF), which are the two functions investigated in this study (Figure 3.1) (Hess & Howell, 1977), Vernier acuity as well as deficiencies in stereopsis (Wallace et al., 2011), spatial distortion (Sireteanu, Lagreze, & Constantinescu, 1993), abnormal spatial interactions (Polat, Sagi, & Norcia, 1997) and impaired contour detection (Kovács, Polat, Pennefather, Chandna, & Norcia, 2000); in addition, global processing of form and motion is altered (Aaen-Stockdale & Hess, 2008; Ho, Taylor, & Loo, 2015; Simmers & Bex, 2004; Simmers, Ledgeway, & Hess, 2005; Simmers, Ledgeway, Hess, & McGraw, 2003).

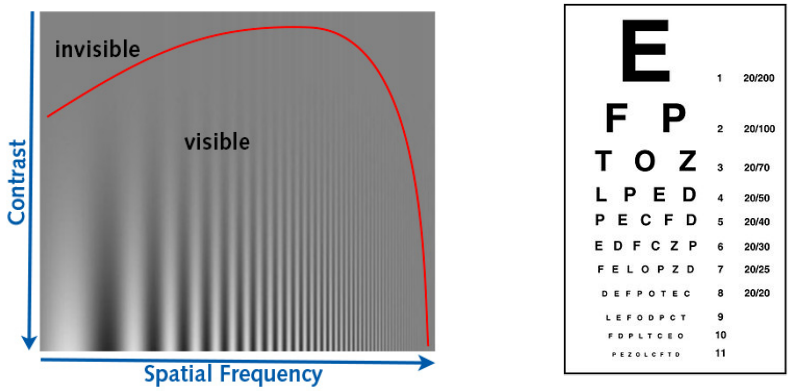


Figure 3.1.: On the left, a canonical image of the contrast sensitivity function. Contrast (grey-level modulation) changes along the vertical axis of this image; spatial frequency (or size) changes along the horizontal axis. The red line marks the boundary between the visible and invisible and it represents a typical contrast sensitivity function in a normal adult. On the right, an example of a standard Snellen chart to measure VA.

Vision consists in a hierarchical processing system, starting from photoreceptors in the retina and extending through several phases of spatial integration in the cortex, each

creating receptive fields of growing complexity resulting in more refined sensory discrimination.

The inter-cortical connections that allow the processing of visual-perceptual information also includes a set of feedback pathways (Figure 3.2) from higher-order frontal areas which provide information for cognitively interpreting visual scenes and for creating a stable representation of the visual scene. Cortical visual neurons are subject to various top-down influences, which depend on attentional processes, perceptual context and expectation (Gilbert & Li, 2013).

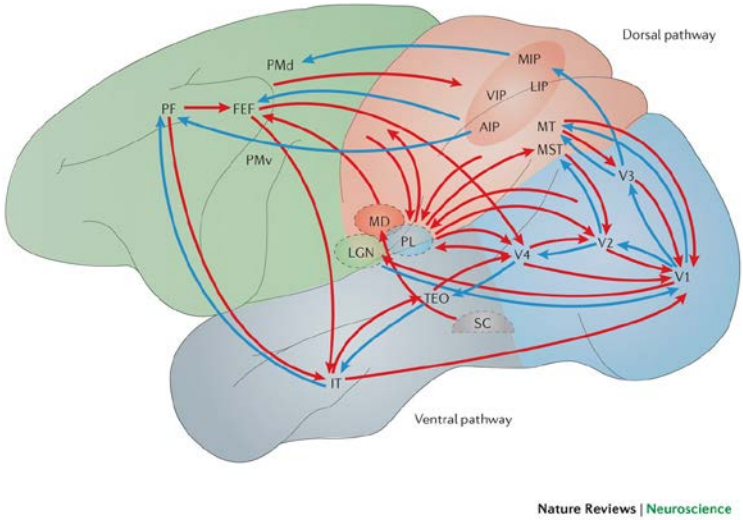


Figure 3.2. Neural connections at cortical and subcortical level involved in visual processing: the blue arrows represent feedforward connections, including the ventral pathway and the dorsal route, which extend from V1 to the prefrontal PF cortex. The red arrows represent feedback connections, through which the higher-order frontal areas influence the early visual areas, and, with them, the sensory processing process (Taken from Gilbert and Li, 2013).

Despite some early indications that the retina may be the primary site of amblyopia (Hess, 2001), the current shared opinions indicate that the V1 is the first site involved in neural loss in amblyopia, manifesting as an atypical pattern of functional

connectivity among neurones selective for orientation and spatial frequency (Polat, 1999; Polat et al., 1997).

A study with functional magnetic resonance imaging (fMRI) supported that visual dysfunction in amblyopia occur within V1 but also in the extrastriate and later specialised cortical areas (V4, lateral occipital complex)(Wong, 2012). Moreover, a reduction of the connectivity of geniculate-striate and striate-extrastriate networks has been shown (Bedny, Konkle, Pelphrey, Saxe, & Pascual-Leone, 2010).

The clinical relevance of amblyopia should not be underestimated because, aside from refractive defects, represents the most frequent cause of vision loss in children, with an incidence of around 2–4% of the population.

Amblyopia was thought to be untreatable if identified after the critical period, that is after ten years (Epelbaum, Milleret, Buisseret, & Duffer, 1993; Greenwald & Parks, 1999), due to aged-diminished neural plasticity within the visual cortex that would limit any anatomical, physiological or functional changes (Berardi, Pizzorusso, Ratto, & Maffei, 2003).

In addition to a specific optical correction that alone can constitute an effective treatment of amblyopia in almost a third of the children treated (Cotter, 2006), two are the treatments mostly recommended within the critical period of the development of the visual system: the first is called patching, which is often adopted, and it consists of bandaging the non-amblyopic eye (patching). Covering the healthy eye induce the visual inputs coming from the amblyopic eye not to be suppressed anymore, therefore the increased stimulation to which this eye is subjected, contributes to the development or recovery of its visual acuity. The second treatment consists in the

pharmacological penalization through the administration of atropine (Wang, 2015). Studies on adult human visual cortex shown manifestations of plasticity, such as perceptual learning (PL), at early levels (Pourtois, Rauss, Vuilleumier, & Schwartz, 2008; Aniek Schoups, Vogels, Qian, & Orban, 2001), reporting improvements associated to several visual functions following perceptual training (Chung, Li, & Levi, 2006; Fiorentini & Berardi, 1981; Karni & Sagi, 1991; Poggio, Fahle, & Edelman, 1992; Sagi, 2011; Schoups, Vogels, & Orban, 1995; Wong, 2012) in healthy adults beyond the critical period.

Perceptual learning

Perceptual learning (PL), a behavioural manifestation of plasticity, is a form of implicit learning, where encoding and retrieval do not require conscious awareness. It may occur practising a challenging task recurrently, resulting in a significant and persistent improvement in the trained perceptual task (Karmarkar & Dan, 2006). The effects of perceptual learning have been well documented beyond the critical period of visual development in healthy adults (Wong, 2012). The mechanisms by which brain plasticity acts are not yet fully known, however, yet it appears clear that changes occur at the synaptic and cellular level, as well as at the level of cortical representations (Levi, 2005).

The investigation of PL has a long tradition (for a review, see Gibson, 1953), which encompass several perceptual domains: auditory, somatosensory and visual ones and as far as the visual domain is concerned, it results being the most investigated. The studies on the neural substrates of PL originated from the observation concerning the specificity of the improvement of perceptual performance, which led to the hypothesis

that PL depended on local changes in V1. The hypothesis on the centrality of V1 in the perceptual learning process is still debated. If on the one hand, several electrophysiological studies (Casco, Campana, Grieco, & Fuggetta, 2004; Hua et al., 2010) and neuroimaging (Furmanski, Schluppeck, & Engel, 2004; Schwartz, Maquet, & Frith, 2002) conducted on adult human subjects showed significant plasticity effects of PL in V1, on the other hand, there is growing evidence for generalization of PL not only to the trained task (Ahissar & Hochstein, 1996; Campana & Casco, 2003; Casco, Campana, & Gidiuli, 2001; Fahle, Friederici, & Ungerleider, 2005; Fiorentini & Berardi, 1981; Karni & Sagi, 1991), but also, under certain conditions, to untrained visual tasks or functions, suggesting the involvement of higher-level areas (Casco et al., 2014; Harris, Gliksberg, & Sagi, 2012; Jeter, Dosher, Petrov, & Lu, 2009; Maniglia et al., 2011, 2016; Mastropasqua, Galliussi, Pascucci, & Turatto, 2015; Solgi, Liu, & Weng, 2013). Likewise, higher non-retinotopic brain areas, for example, the frontoparietal, have demonstrated to be involved in attentional and decision-making processes (Gold & Shadlen, 2007; Mukai et al., 2007; Schwartz et al., 2005; Xiao et al., 2008), exercising a top-down control on early visual areas (Figure 3.2).

Perceptual learning and amblyopia

In the last twenty years, studies reported that through practice, consisting in a repeated and demanding visual task (e.g. contrast detection) marked improvements of various visual functions in adults with amblyopia occurred (Astle, McGraw, & Webb, 2011; Chung et al., 2006; Levi & Li, 2009; Li, Young, Hoenig, & Levi, 2005; Polat, 2009; Polat, Ma-Naim, Belkin, & Sagi, 2004; Zhou et al., 2006).

Perceptual training is usually performed monocularly, thus with the concomitant occlusion of the non-amblyopic eye through patching. As mentioned, several types of visual tasks showed significant improvement with practice: for instance, position acuity (Li & Levi, 2004), letters recognition (Levi, 2005), contrast detection, in particular, grating detection (Polat et al., 2004; Zhou et al., 2006).

Zhou et al. (2006) conducted a study with three different perceptual training with adults with anisometric amblyopia. The training consisted of a single Gabor detection located at the centre of the screen. The first group trained on grating detection in the amblyopic eye, using each individual's pre-training cut-off spatial frequency, the second group received training at varying spatial frequencies in the amblyopic eye and the third group did not receive any training. Results of this study indicated that training improved VA and CSF in the amblyopic eye of all the participants in groups I and II (the most significant improvements were seen in group I), whereas no significant improvement in performance has been observed in group III. Maintenance of VA result was observed in a few of the cases tested for up to one-year post-training (Zhou et al., 2006). This is a valuable study suggesting that the adult amblyopic brain might still be capable of plasticity and recovery of function.

According to Levi and Li (2009) and Camilleri and colleagues (2014), contrast detection task using the lateral masking paradigm was able to produce the major improvement ratio on both VA and contrast sensitivity (CS) functions (Polat et al., 2004; Polat & Sagi, 1993). Polat and colleagues (2004) used a training procedure based on the strengthening of facilitatory lateral interactions and a weakening of inhibitory lateral interactions between detectors tuned to specific orientations and spatial frequencies,

by administering a two-interval forced choice (2IFC) contrast-detection task performed monocularly (with the amblyopic eye), consisting of a low-contrast central Gabor patch flanked by two high-contrast Gabor patches (Figure 3.3). Each training session consisted of 10–15 blocks (from 30 to 80 sessions) with different target flanker distances, 1.5 λ, 3 λ, 4 λ, 8 λ (the wavelength of the Gabor stimulus), and different spatial frequencies, moving progressively from lower to higher ones, in four orientation (0°, 45°, 90°, 135°) for each spatial frequency. Results showed that this training produced an improvement of contrast-detection thresholds (ranging from 2.05 to 4.23 times), and improvement in VA (78% gain, equal to 0.25 LogMAR).

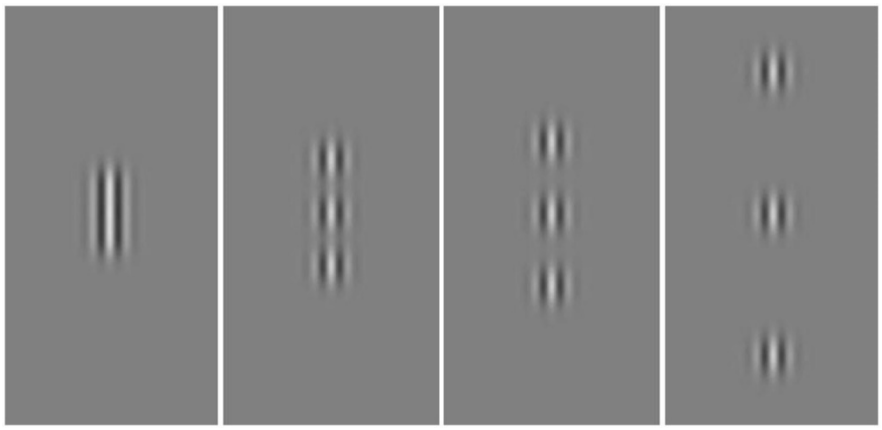

Figure 3.3. Gabor stimuli used during perceptual training. The central stimulus (target) varies in its contrast according to the participant's performance and always appears centrally. The two flankers, collinear with the target, have a fixed contrast value. Proceeding from left to right, the target-flankers distance varies, assuming the following values: 1.5, 3, 4, 8 λ.

The conserved improvement of visual function after treatment, showing that the learning was more than a temporary modulation, supporting long-term cortical plasticity in human adults (Chung et al., 2006; Li et al., 2005; Polat et al., 2004; Zhou et al., 2006).

NIBS and visual functions

In the last years, an additional method that has been investigated for enhancing visual functions in amblyopia consists of the administration of NIBS techniques over visual areas.

Regarding tES techniques, the first study to explore the effects of tDCS on visual perception, specifically in contrast perception, demonstrated that 7 minutes of c-tDCS diminished the excitability and reduced contrast perception, whereas a-tDCS did not result in any significant cortical or behavioural modulations (Antal, Nitsche, & Paulus, 2001). The differences obtained might be attributed to both different stimulation protocols, in fact, tDCS is polarity-dependant thus depending on whether cathodal or anodal stimulation is administered, the effect may be opposed (see Chapter 1, tDCS section) (Nitsche et al., 2008).

Other recent studies have shown that the combination of a-tDCS applied on the occipital cortex with visual field rehabilitation seemed to enhance visual functional performance compared with visual rehabilitation alone (Plow, Obretenova, Fregni, Pascual-Leone, & Merabet, 2012; Plow et al., 2011).

In human adults, it has been demonstrated that a single session of a-tDCS over the visual cortex during a contrast-detection or discrimination task, yielded a temporary improvement in contrast sensitivity in the amblyopic (Ding et al., 2016; Spiegel, Byblow, Hess, & Thompson, 2013) and fellow eye (Ding et al., 2016).

Spiegel and colleagues (2013) using fMRI, reported that following a-tDCS, a reduction in the visual cortical response asymmetry in amblyopic patients, favouring the fellow eye (Spiegel et al., 2013a). The same research team, showing that five sessions of

dichoptic training with concurrent a-tDCS over the occipital cortex produced a larger improvement of stereopsis (but not visual acuity) with respect to the same dichoptic treatment with concurrent Sham stimulation (Spiegel, Li, et al., 2013).

Moreover, a recent animal study using tDCS demonstrated that in monocular rats deprived from birth, eight sessions of a-tDCS on the visual cortex contralateral to the deprived eye produced an almost complete recovery of visual acuity (Castaño-Castaño et al., 2017).

A reduction of GABA following a-tDCS application has been reported with magnetic resonance spectroscopy (MRS) (Stagg et al., 2009).

The theorised effect of brain stimulation on visual functions has been attributed to disinhibition of the suppressed processing of information arriving from the amblyopic eye, likely mediated by a reduction of the concentration of the inhibitory neurotransmitter GABA (Ding et al., 2016). Such a reduction of GABA could also explain the boosting of learning when tDCS was coupled with a visual task (Sale, Berardi, Spolidoro, Baroncelli, & Maffei, 2010). This GABA decrease has found out to positively correlate with improvement in motor learning performance and negatively correlate with the change in BOLD signal in the motor cortex (Kim, Stephenson, Morris, & Jackson, 2014).

Other studies focusing on TMS technique, shown an increase in CS for high spatial frequencies after a single session of rTMS delivered over the visual cortex; a study reported that five sessions of cTBS, a protocol causing cortical inhibition (see chapter 1, TMS paragraph), produced a long-term improvement of CS for high spatial frequencies in the amblyopic eye that lasted up to 78 days (Clavagnier, Thompson, &

Hess, 2013). The results showed that cTBS while subjects observe a high-contrast (grating) stimulus with the non-amblyopic eye, significantly improved the contrast sensitivity (for high spatial frequencies) of the amblyopic eye, with a cumulative effect among the various sessions. This task was performed with the occluded amblyopic eye basing on the hypothesis that an inhibition mechanism, if applied to neural populations per se inhibited or suppressed, such as those that respond to visual inputs coming from the amblyopic eye, causes an increase in neural excitability (Silvanto, Cattaneo, Battelli, & Pascual-Leone, 2008).

TRNS and perceptual learning

TRNS, the most recent tES technique (see chapter 1, tRNS paragraph), has been shown, using the high-frequency range, to be the most efficient neuromodulatory technique for enhancing and accelerating PL during a training (Fertonani et al., 2011; Pirulli et al., 2013). Fertonani and colleagues (2011) revealed that different stimulation conditions had a distinctive effect on the learning effect seen during task execution and on the resulting performance. Their results showed the efficacy of hf-tRNS at 1.5 mA online, administered for a total duration of ~ 20 min per session, in boosting PL on a visual discrimination task. Pirulli and colleagues (2013) have shown that tRNS facilitated PL only if applied online, thus simultaneously with the visual task.

A pilot study by Campana and colleagues (2014), which combined 8 sessions of hf-tRNS together with a contrast detection training under lateral masking conditions (Polat et al., 2004), reported a considerable improvements in CS function (60% mean improvement averaged across all spatial frequencies) following the training, both in

the trained amblyopic eye and in the untrained fellow eye. Besides, an improvement of mean VA of 0.18 LogMAR (53% mean improvement) in the trained amblyopic eye was found. The specific role of hf-tRNS could not be established in such improvement of visual functions, due to the absence of the Sham condition group.

In light of the previous study which showed the combined effect of tRNS with a perceptual training and, having previously shown that the whole frequency spectrum of hf-tRNS is the most effective in enhancing cortical activity (Chapter 2), in the present study, we propose a short perceptual training (8 sessions) combined with hf-tRNS or Sham stimulation. All participants were randomly assigned to either the hf-tRNS or Sham group, both groups performing a behavioural training regime using the lateral masking paradigm (Campana, Camilleri, Pavan, Veronese, & Giudice, 2014; Polat et al., 2004). We theorised that hf-tRNS could boost and quicken the effects of PL when combined with a short perceptual training paradigm in adults with amblyopia. Moreover, we hypothesised that by boosting the rate of PL via the modulation of neuronal plasticity, hf-tRNS might be successfully used to reduce the number of sessions of the perceptual training. To explore the effects of online hf-tRNS, VA and CSF were assessed for each observer and each eye before and after the training.

## 3.2. Methods

Participants

Twenty amblyopic patients (mean age 44 years, ranging from 27 to 58) were recruited at the San Paolo Ophthalmic Center of San Antonio Hospital (Padova, Italy). To diagnose amblyopia, a difference in interocular VA of at least 0.1 LogMar (2/10) with

VA in the amblyopic eye equal or greater than 0.1 LogMar and an onset occurred in childhood were required. The best optical correction for each patient was assessed and provided approximately a month before enrolling in the study. A power analysis showed that in order to get an effect size of 0.8 (i.e., large effect size) a sample of six participants could have been used. In the present study, a sample of 10 participants per group was employed in order to get an effect size of ~0.99 on VA.

Table 3.1 reported the characteristics of each amblyopic patient. The participants were randomly divided into two groups, ten participants for each group. Both groups underwent a short (8 sessions) contrast-detection behavioural training using the lateral masking paradigm (Polat, 2009; Polat et al., 2004). Participants in the first group performed the training combined with online hf-tRNS, whereas participants in the second group (controls), performed the same training combined with Sham stimulation. Exclusion criteria included blindness in one eye, VA below 0.1 LogMAR in the amblyopic eye, any ocular condition or cause for reduced VA other than amblyopia, myopia, presbyopia, hypermetropia and/or astigmatism; these include diabetes mellitus, pregnancy, the presence of myopia-related ocular complications and any previous ocular surgery. Exclusion criteria also included incompatibility with transcranial electrical stimulation, as assessed by a questionnaire (e.g., history of seizures, skin problems, migraine, etc.). After completing the experimental research training, participants in the Sham control group were offered the opportunity to take part in a re-training with hf-tRNS administration. Besides, all participants gave written informed consent according to the Declaration of Helsinki. This study has been approved by the local Ethics Committee (Protocol Number: 1248).

| Hf-tRNS group | N | Gender | Age | Amblyopic Eye | Sph RE | cyl RE | ax RE | Sph LE | cyl LE | ax LE | VA RE | VA LE | CS RE | CS LE | Training SF (cpd) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | m | 30 | LE | 1.5 | -4.5 | 170 | 1,5 | -4.5 | 173 | 0.10 | 0.30 | 447 | 316 | 6 |
| | 2 | f | 48 | RE | -6.5 | -2 | 180 | 0.5 | -1 | 9 | 0.10 | 0.00 | 224 | 224 | 9 |
| | 3 | f | 56 | LE | -2.25 | -2 | 20 | -6 | -3.5 | 178 | 0.00 | 0.30 | 447 | 224 | 3 |
| | 4 | f | 45 | LE | 1.75 | -0.5 | 145 | 3.75 | -2.25 | 20 | -0.10 | 0.20 | 891 | 891 | 6 |
| | 5 | m | 55 | RE | 1 | / | / | 0.5 | / | / | 1.00 | 0.10 | 56 | 447 | 1.5 |
| | 6 | m | 27 | RE | 5 | -5.75 | 178 | / | -0.5 | 162 | 0.20 | -0.10 | 316 | 891 | 9 |
| | 7 | f | 32 | RE | -7 | -1.5 | 142 | / | -0.5 | 180 | 0.50 | -0.10 | 158 | 447 | 6 |
| | 8 | f | 55 | RE | 1.25 | 2.25 | 45 | -0.25 | -1 | 70 | 0.50 | -0.10 | 631 | 891 | 9 |
| | 9 | f | 48 | RE | -8 | -0.25 | 130 | -0.75 | -0.5 | 100 | 0.20 | -0.10 | 447 | 891 | 6 |
| | 10 | m | 30 | LE | 2.75 | 1.5 | 57 | 3.75 | 1.75 | 100 | 0.10 | 0.50 | 891 | 891 | 6 |
| Sham group | | | | | | | | | | | | | | | |
| | 11 | f | 52 | RE | -6 | -4.5 | 5 | -5 | -5.75 | 10 | 0.20 | 0.10 | 316 | 447 | 12 |
| | 12 | f | 48 | RE | -8 | -0.25 | 130 | -0.75 | -0.5 | 100 | 0.40 | 0.10 | 447 | 891 | 6 |
| | 13 | m | 28 | LE | / | / | / | 0.5 | 3.5 | 95 | -0.10 | 0.50 | 447 | 447 | 9 |
| | 14 | f | 41 | LE | -0.75 | -0.25 | 123 | -0.25 | -0.25 | 18 | 0.00 | 0.30 | 891 | 447 | 12 |
| | 15 | m | 30 | LE | 2.75 | 1.5 | 57 | 3.75 | 1.75 | 100 | 0.40 | 0.60 | 447 | 447 | 3 |
| | 16 | f | 55 | RE | 4.25 | -0.5 | 14 | 2 | -0.25 | 130 | 0.30 | 0.00 | 447 | 891 | 6 |
| | 17 | m | 49 | RE | -2 | -2.5 | 24 | -2.5 | -1.5 | 152 | 0,40 | 0.20 | 631 | 891 | 9 |
| | 18 | m | 48 | LE | 0.5 | 0.5 | 180 | 0 | 3.75 | 90 | -0.10 | 0.20 | 891 | 891 | 9 |
| | 19 | f | 52 | LE | 0.5 | 0.25 | 3 | 2.25 | 1 | 107 | -0.20 | 0.60 | 891 | 447 | 3 |
| | 20 | f | 58 | LE | 3.25 | 0.75 | 160 | 3.75 | 0.25 | 145 | 0.00 | 0.80 | 224 | 158 | 3 |

Table 3.1. Characteristics of amblyopic patients. The following correction parameters are reported: *sph* (spherical correction), indicating the amount of lens power, in diopters, to correct nearsightedness or farsightedness (negative numbers indicate nearsightedness, positive numbers farsightedness), *cyl* indicates the amount of lens power for astigmatism, *ax* (axis, present only if there is a value for *cyl*), indicating the angle (in deg) of one of two major meridians where the cylindrical power is in. VA indicates initial visual acuity (in LogMAR) and CS is the initial contrast sensitivity (in (1/ Weber contrast) *10) as measured at the St. Antonio Hospital with ETDRS and Pelli-Robson contrast sensitivity chart respectively. The last column reports the spatial frequency (in cycles per degrees; cpd) of the Gabor patches used in the experiment (see the Stimuli and Procedure section).

## Apparatus

A battery-driven stimulator (BrainSTIM, EMS) and two electrodes inserted into physiological saline-solution soaked sponges were used to deliver the stimulation.

A 22-inch screen (Philips Brilliance 202P4) with a resolution of 1280 x 1024 pixels and with a refresh rate of 60 Hz has been used for both the VA assessment and perceptual training. The screen luminance was calibrated by gamma correction, with γ = 1. Screen luminance was calibrated using Spyder 5 Express (Datacolor, Lawrenceville, New Jersey, USA; http://www.datacolor.com/). The luminance of the screen background was fixed at 31.5 cd/m². CS was measured with a computer equipped with a VSG2/3

graphic card (Cambridge Research Systems Ltd) with 12-bit luminance resolution. Stimuli were presented on a 17-inch CRT monitor (Philips Brilliance 107P), with resolution of 1024 x 768 pixels and a refresh rate of 70 Hz. The stimuli were generated using CRS Psycho 2.36 test (Cambridge Research Systems Ltd, Rochester, UK). The luminance of the background of this screen was fixed at 48.5 cd/m², and the screen luminance was kept with γ = 1. All tests and the training were carried out in a dark and silent room.

Assessment

VA was tested before and after the behavioural training using the Landolt-C test of the Freiburg Acuity and Contrast Test 3.8 (FrACT 3.8; Bach, 1996) at a viewing distance of 3 m. With the screen configuration and the viewing distance used, a pixel subtended ~0.34 arcmin. The Landolt-C test consists of an orientation discrimination task, with eight possible choices corresponding to eight positions of the gap in the stimulus used. The stimulus, the letter C, remained visible on the screen until the participant's response. Landolt-C stimuli were uncrowded. The appearance of the stimulus was accompanied by an auditory signal, while a different auditory signal was used for the wrong answers. The Best PEST adaptive procedure (Pentland, 1980) was used to determine the VA threshold corresponding to 62.5% of discrimination accuracy.

Contrast sensitivity was measured before and after the training using the method of limits. In particular, we presented a vertical sinusoidal grating covering the whole screen area (21.3° x 16°) at a distance of 1.5 m from the screen using the CRS Psycho test 2.36 (Cambridge research System Ltd, Rochester, UK). In the main CSF experiment, participants were required to complete four ascending series in which the contrast of

the sinusoidal grating varied from high to low levels, and four descending series in which the contrast varied from low to high levels. The procedure always started from a descending series, followed by alternating series.

The initial contrast of the first descending series was set based on a pilot experiment and was -16dB for low and intermediate spatial frequencies (i.e., 0.8, 2.9 and 5.8 cycles per degree) and -9dB for high spatial frequencies (i.e., 9.7 and 14.5 cycles per degree). On successive series, the initial contrast was set as the contrast threshold obtained in the previous series, plus (in descending series) or minus (in ascending series) a factor between 6dB and 10dB randomly chosen. Increments and decrements were equal to 2dB. The final contrast threshold was calculated for each spatial frequency by averaging the contrast threshold estimated for each of the eight series. This procedure was repeated for each spatial frequency, with spatial frequencies presented sequentially starting from the lowest one.

*Experimental Procedure*

Following the pre-training tests, the participants performed eight sessions of behavioural training consisting of a two-interval forced choice task (2IFC) where the participant has to press one of two designated keys on a standard Italian keyboard depending on whether the target appeared in the first ('Z') or second interval ('M') of the stimulus sequence. The training lasted four consecutive days per week for two weeks. Each session included eight blocks of maximum 60 trials each (i.e., ~5/6 minutes per block; ~45 minutes per session). The stimuli used were Gabor patches, consisting of a vertical sinusoidal grating enveloped by a Gaussian window with the standard deviation (σ) equal to the sinusoidal wavelength (λ), so the size of the Gabor

stimuli varied with the spatial frequency of the grating (Polat and Sagi, 1993). The contrast of the target Gabor varied depending on the participant's performance according to a 1 up / 3 down staircase (Levitt, 1971) and was calculated averaging the last eight reversals of the staircase, corresponding to 79% of correct detection. The staircase terminated either after 60 trials or 18 reversals. Two high-contrast Gabor patches with 0.6 Michelson contrast, collinear to the target and with the same spatial frequency, were presented (i.e., flankers). Stimulus duration was 200 ms and its appearance was always accompanied by an acoustic signal (temporal cue) and a central fixation point (spatial cue). Stimuli were presented at the centre of the screen. The distance between the centre of the target and the centre of the flanker stimuli varied every two consecutive blocks. The target-to-flankers distance was measured in multiples of the sinusoidal carrier's wavelength: 1.5 λ, 3 λ, 4 λ, and 8 λ (Figure 3.4 A). The orientation of the stimulus configuration also varied every two consecutive days of the training, from 0° (vertical), then 45°, 90° [horizontal] and 135°, finally (Figure 3.4 B). The trained spatial frequency was chosen, for each participant, based on the cut-off performance in the pre-training CSF, that is the highest spatial frequency with contrast threshold approximately equal to 0.50 Michelson contrast (Camilleri et al., 2014, 2016; Zhou et al., 2006). The range of trained spatial frequencies in the sample of participants ranged from 3 to 12 cpd. In addition, follow-up of VA measurements was carried out 6 months after the training, in order to verify the long-term effects of the combination of perceptual training and non-invasive electrical stimulation.

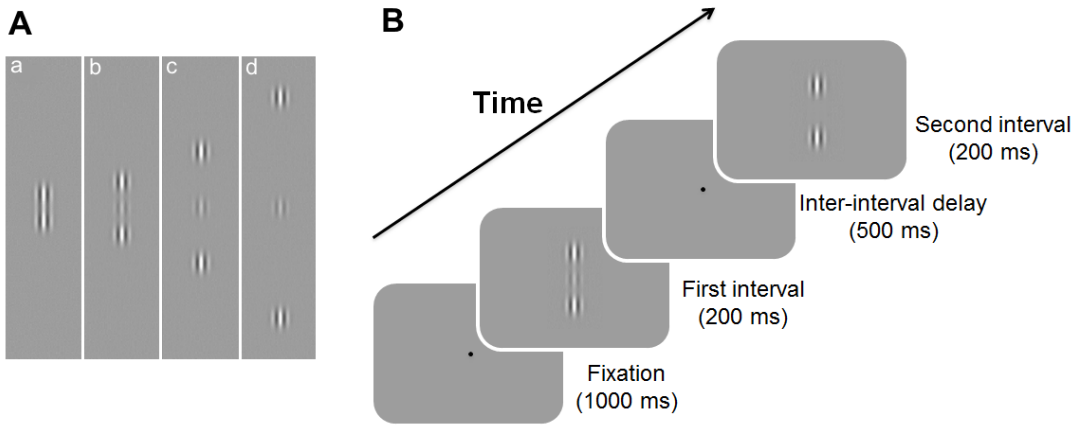


Figure 3.4. Representation of the stimuli and procedure used in the training sessions. (A) A vertical Gabor patch (target) was flanked above and below by two high contrast Gabor patches (flankers). Panel (a) target-to-flankers distance of 1.5 λ, panel (b) 3 λ, panel (c) 4 λ, and panel (d) 8 λ. (B) Schematic representation of the procedure used in the training sessions. After an initial fixation of 1 s, two temporal intervals were presented. In the first interval (200 ms), the target is flanked above and below by two high-contrast Gabor patches of the same frequency and at a target-to-flankers distance of 3λ. After a delay of 500 ms, a second interval is presented and contained only the flankers. The task was to detect in which of the two temporal intervals was presented the target patch. The Gabor patches represented have a spatial frequency of 3 cpd. The contrast of the central patch (i.e., the target) has been increased for demonstrative purposes.

During the first 5 blocks, high-frequency random noise current (ranging from 100 Hz to 600 Hz), was delivered with a current intensity of 1.5 mA and 0 mA offset. Current linearly increased in intensity up to 1.5 mA during the first 30 s of stimulation. The current density was maintained within the safety limits (i.e., below 1.0 $A/m^2$; Poreisz, Boros, Antal, & Paulus, 2007). One electrode, with an area of 16 $cm^2$, was placed on the occipital cortex, with the centre at ~3 cm above the inion, while the other electrode (reference) with an area of 60 $cm^2$, was positioned on the participant's forehead. The electrodes were kept in place with bandages. In order to keep the total duration of stimulation within 25 minutes, hf-tRNS was applied to the first 5 blocks of each training session, with each block lasting ~5/6 min. During the last 3 blocks, no

electric stimulation was delivered (Fertonani et al., 2011). In the Sham group, the current linearly increased for the first 30 s up to a 1.5 mA and then decreased to 0 mA in the next 30 s. Training and stimulation protocols were set by a researcher and another researcher carried out the pre- and post-training assessments.

## 3.3. Results

*Contrast Sensitivity*

We performed a mixed ANOVA including Training (pre-training vs post-training), Eye (amblyopic/trained vs non-amblyopic/untrained) and Spatial Frequency (0.8, 2.9, 5.8, 9.7, and 14.5 cpd) as within-subjects factors and Group (hf-tRNS vs Sham) as the between-subjects factor. When the sphericity assumption was violated, degrees of freedom were corrected with the Greenhouse-Geisser correction. The ANOVA did not show any effect of the Group ($F_{1,18}$ = 0.101, $p$ = 0.754, $\eta^2_p$ = 0.006). CS significantly improved after training ($F_{1,18}$ = 19.088, $p$ = 0.0001, $\eta^2_p$ = 0.515) in both groups. The interaction Training x Group was not significant ($F_{1,18}$ = 0. 910, $p$ = 0.353, $\eta^2_p$ = 0.048) indicating that both groups improved in a similar way. The difference between trained and untrained eye (i.e., amblyopic vs. non-amblyopic) was also significant ($F_{1,18}$ = 50.980, $p$ = 0.0001, $\eta^2_p$ = 0.739). The interaction Eye x Group was not significant ($F_{1,18}$ = 1.368, $p$ = 0.257, $\eta^2_p$ = 0.071). There was a significant difference in CS between the different spatial frequencies tested ($F_{1.56,\ 28.16}$ = 19.137, $p$ = 0.0001, $\eta^2_p$ = 0.515). The interaction Spatial Frequency x Eye was significant ($F_{2.15,\ 38.73}$ = 7.095, $p$ = 0.002, $\eta^2_p$ = 0.283) suggesting that the two eyes have different CS at specific spatial frequencies. Moreover, the interaction Training x Spatial Frequency was also significant ($F_{2.24,\ 40.41}$ =

3.510, $p$ = 0.035, $\eta^2_p$ = 0.163) suggesting that the CS improved at certain spatial frequencies mainly. We then compared pre- vs post-training measurements for each spatial frequency with paired-sample t-tests using a false discovery rate (FDR) at 0.05 for multiple comparisons (Benjamini & Hochberg, 1995; Benjamini & Yekutieli, 2001) to explore the interaction between training and spatial frequency. Post-hoc comparisons reported a significant improvement for all the spatial frequencies employed (*critical-p* = 0.048) (Figure 3.7).


Figure 3.7. CS results (Logarithmic scale). (A) CS curves for the hf-tRNS group, for trained and untrained eyes. (B) CS curves for the Sham group, for trained and untrained eyes. Error bars ±1SEM

Moreover, the magnitude of CS improvement from the pre-training to the post-training sessions for each participant of the two groups (i.e., hf-tRNS and Sham), and for each eye (amblyopic/trained vs non-amblyopic/untrained) was calculated. Following Zhou et al. (2006), the magnitude of CS improvement ($CS_I$) was calculated in dB using the following equation:

$$CS_I = 20log_{10}\frac{CS_{post-training}}{CS_{pre-training}} \quad \text{Eq. 1}$$

The magnitude of CS improvement was then converted to percentage increment ($P_I$) as follow:

$$P_I = 100\left(10^{CS_I/20} - 1\right) \quad \text{Eq. 2}$$

We performed a mixed ANOVA on the magnitude of CS improvements with the Eye (trained vs untrained) and Spatial Frequency as within-subject factors, and the Group (hf-tRNS vs Sham) as a between-subject factor. No significant effect or interaction was found. The same analysis was performed on the CS percentage increments (Table 3.2), and any significant effect or interaction was found.

| | | Spatial Frequency (cpd) | | | | |
|---|---|---|---|---|---|---|
| | | **0.8** | **2.9** | **5.8** | **9.7** | **14.5** |
| **hf-tRNS** | **Trained eye** | 79.54 (29.78) | 73.57 (20.48) | 74.97 (18.51) | 101.92 (19.89) | 215.93 (67.56) |
| | **Untrained eye** | 71.09 (33.86) | 21.23 (7.02) | 69.22 (16.08) | 57.53 (24.31) | 61.37 (27.76) |
| **Sham** | **Trained eye** | 59.97 (28.33) | 47.37 (15.94) | 87.66 (53.88) | 82.99 (37.60) | 166.20 (113.77) |
| | **Untrained eye** | 22.22 (18.88) | 57.08 (24.82) | 63.88 (35.40) | 115.27 (52.51) | 51.49 (23.71) |

Table 3.2. CS percentage improvement. Percentage CS increment (SEM in %) for the two groups (hf-tRNS and Sham) and for the trained and untrained eyes.

These results suggest that hf-tRNS did not increase CS during the training sessions when compared with the Sham group. However, there was a general effect of the perceptual learning that generalised to the untrained eye and was similar in both groups (Figure 3.7).

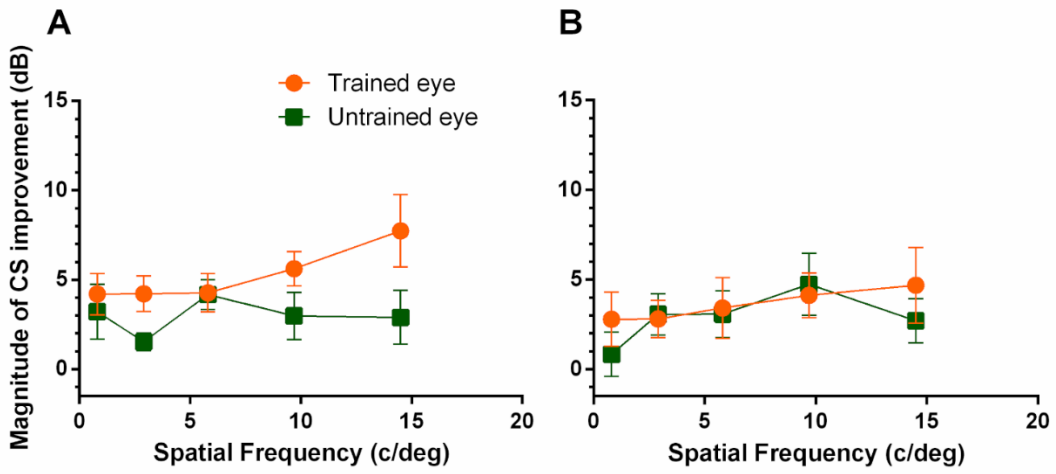


Figure 3.7. CS improvement. Mean magnitude of CS improvement (dB) for the hf-tRNS group (A) and the Sham group (B). The two eyes (trained and untrained) are represented by separate lines. Error bars ±SEM.

### *Visual Acuity*

To verify any difference in VA impairment at the baseline between hf-tRNS group and Sham group, we performed a mixed ANOVA including Group (hf-tRNS vs Sham) as a between-subjects factor and Eye (amblyopic vs. non-amblyopic) as a within-subjects factor. The ANOVA did not reveal any significant difference between the two groups ($F_{1,18}$ =1.06, $p$ = 0.317, $\eta^2_p$ = 0.056), nor any significant interaction between Group and Eye ($F_{1,18}$ = 0.836, $p$ = 0.373, $\eta^2_p$=0.044), confirming that the two groups had similar VA before training.

A mixed ANOVA was used to compare pre- and post-training measurements of VA with the Group as a between-subjects factor and Training (pre- vs. post-training) and Eye (trained vs. untrained) as within-subjects factors. A significant interaction between Training x Group ($F_{1,18}$ = 6.445, $p$ = 0.021, $\eta^2_p$ = 0.264) indicated that the groups differed in their pre- vs. post-training VA measurements. We then performed a further analysis separately for each group to understand how each group improved VA.

For the hf-tRNS group, a repeated-measures ANOVA including Eye (trained vs. untrained) and Training (pre-training, post-training and follow up) as within-subjects factors, revealed a significant difference between the trained and the untrained eye ($F_{1,18}$ =34.831, $p$ = 0.0001, $\eta^2_p$ = 0.795), due to the evident difference in VA between the amblyopic eye and the non-amblyopic eye. Pre- and post-training measurements were significantly different ($F_{2,18}$ = 28.921, $p$ = 0.0001, $\eta^2_p$ = 0.763), with no significant interaction between Training and Eye ($F_{2,18}$ = 2.026, $p$ = 0.161, $\eta^2_p$ =0.184), indicating that both trained and untrained eyes improved similarly. Post-hoc t-tests using an FDR at 0.05 and pooling data from the two eyes, showed a significant difference between pre- and post-training in the hf-tRNS group ($t_9$ = 7.187, $p$ = 0.0001) and a significant difference between pre-training and follow-up ($t_9$ = 5.408, $p$ = 0.001), indicating that VA improvement was maintained after six months (Figure 3.5 A). The mean improvement at post-training was equal to 0.19 LogMAR for the amblyopic eye, and 0.11 LogMAR for the non-amblyopic eye. The power analysis performed on the hf-tRNS group, for the trained eye and between the pre and post-training sessions, reported a value of ~0.99.

Regarding the Sham group, a repeated-measures ANOVA including Eye (trained vs. untrained) and Training (pre- vs. post-training) as within-subjects factors revealed no significant difference between pre- and post-training measurements ($F_{1,9}$ = 1.156, $p$ = 0.310, $\eta^2_p$ = 0.114), indicating the absence of a VA improvement in the Sham group (Figure 3.5 B).

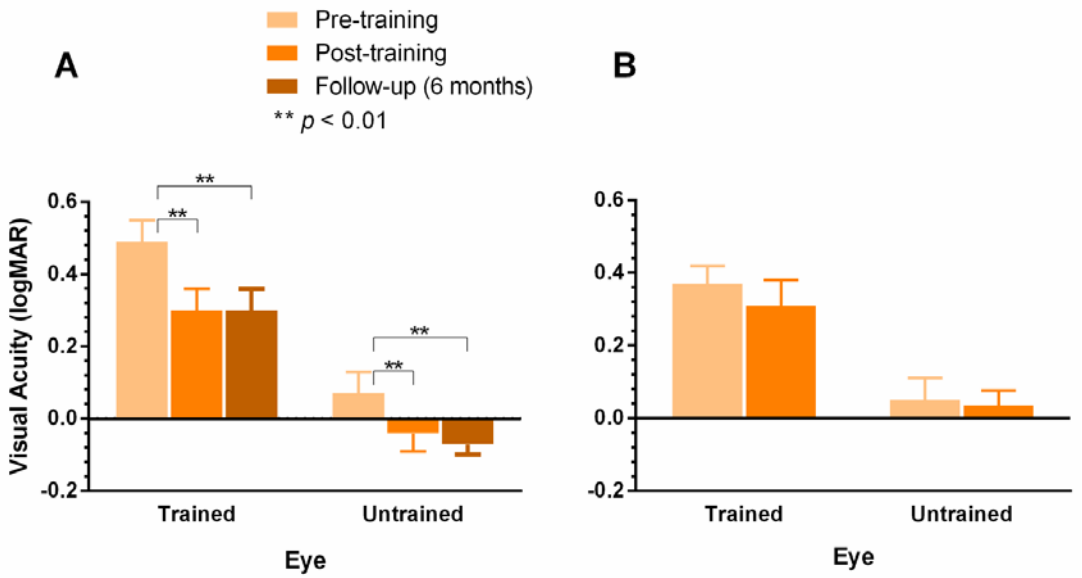


Figure 3.5. Visual Acuity results (LogMAR). (A) VA for the hf-tRNS group for the trained and untrained eye. (B) VA for the Sham group for the trained and untrained eye. Error bars ±1SEM

In order to obtain a measure of the relative improvement of both the trained and untrained eyes for the two groups, we calculated the difference between post- and pre-training LogMAR VA for the trained and untrained eyes for each group. Values below zero indicate better VA in the post-training section (i.e., lower logMAR VA measure in the post-training sessions), values above zero indicate worse VA in the post-training section (i.e., higher logMAR VA measure in the post-training session), and zero indicates no modulation. For the tRNS group, the mean difference for the trained eye was -0.193 (SEM: 0.022), whereas for the untrained eye was -0.114 (SEM: 0.0259). For the Sham group, the mean difference for the trained eye was -0.068 (SEM: 0.052), whereas for the untrained eye was -0.015 (SEM: 0.037). A mixed ANOVA on the differences between post- and pre-training logMAR VA measures and including as a between-subjects factor the Group (tRNS vs. Sham) and as a within-subjects factor the Eye (trained vs. untrained), revealed a significant effect of the Group ($F_{1,18}$ = 6,44, $p$ = 0.021, $\eta^2_p$ = 0.26), a significant effect of the Eye ($F_{1,18}$ = 5,74, $p$ = 0.028, $\eta^2_p$ = 0.24), but

not a significant interaction between Group and Eye ($F_{1,18}$ = 0,22, $p$ = 0.64, $\eta^2_p$ = 0.012). In order to test for differences between the trained and untrained eye, we performed a paired-sample t-test separately for each group. For the tRNS group, we found a significant difference between the trained and the untrained eye, with greater improvement for the trained eye ($t_9$ = -2.76, $p$ = 0.022, *Cohen's d* = 0.87). On the other hand, for the Sham group, there was a no significant difference between the trained eye and the untrained eye ($t_9$ = -1.26, $p$ = 0.29, *Cohen's d* = 0.36). These results suggest that hf-tRNS improved mainly for the trained eye.

In Figure 3.6, VA (LogMAR) measured in the post-training condition is reported as a function of VA measured in the pre-training condition. For the hf-tRNS group, most of the VA values fall below the diagonal line (black dotted line) indicating larger values in the pre-training (i.e., worse VA) than in the post-training session test. For the Sham group, instead, VA values lie approximately on the diagonal line, indicating no VA modulation. This trend was confirmed by fitting a linear function to the VA data points separately for the hf-tRNS and the Sham groups, and testing with an *F* test whether the intercept was significantly different from zero, and the slope significantly different from 1.0 (i.e., the intercept and slope expected for the diagonal equity line). Linear fits were performed considering trained and untrained eyes together; that is, the linear fit was performed on 20 VA data points for the hf-tRNS group and 20 data points for the Sham group. Linear fits and *F* tests were performed using GraphPad Prism v6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com).

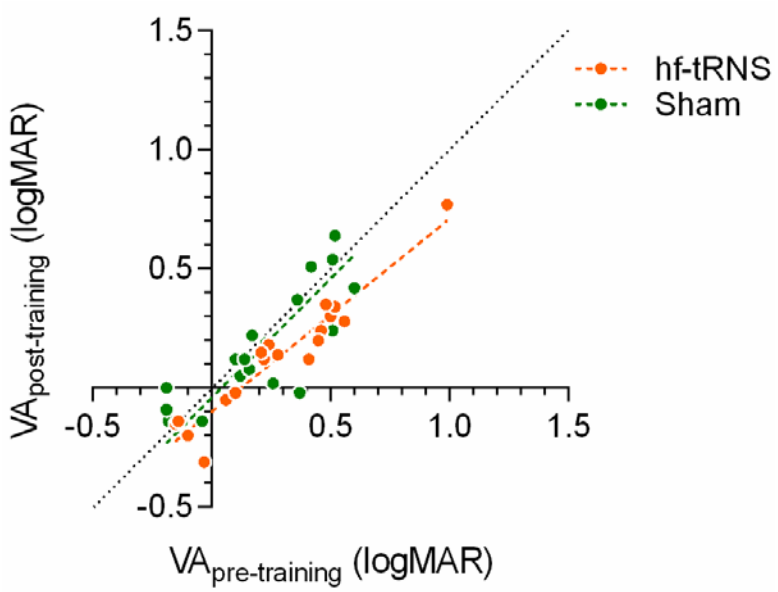


Figure 3.6. Individual VA data. Individual VA data points (LogMAR) for hf-tRNS (orange points) and Sham (green points) groups (data points for the trained and untrained eye are reported together). The black dotted diagonal line indicates the equity line, i.e. when there is no difference in VA between pre- and post-training sessions. The orange dashed line represents the linear fit to VA data points for the hf-tRNS group, whereas the green dashed line represents the linear fit for the Sham group.

For the hf-tRNS group, the intercept was -0.1001 (SE: 0.023) and the slope 0.8097 (SE: 0.058) ($R^2$ = 0.92). An *F* test reported that the intercept was significantly lower than zero ($F_{1,18}$ = 19.21, $p$ = 0.0004) and that the slope was significantly different from 1.0 ($F_{1,18}$ = 10.87, $p$ = 0.004). These results suggested a dramatic improvement of VA when hf-tRNS was applied during the training, and that the amount of improvement was proportional to the initial deficit, with a Weber fraction equal to 0.55. For the Sham group, the intercept was 0.0081 (SE: 0.041), and the slope was 0.766 (SE: 0.128) ($R^2$ = 0.67). An *F* test reported that the intercept was not significantly different from zero ($F_{1,18}$ = 0.04, $p$ = 0.844) and the slope was not significantly different from 1.0 ($F_{1,18}$ = 3.316, $p$ = 0.085). An additional *F* test reported that the two datasets were significantly different ($F_{2,36}$ = 4.12, $p$ = 0.024). These results suggest that VA improvement only occurred if hf-tRNS (not Sham stimulation) was applied during the training.

We also calculated individually for each participant belonging to hf-tRNS group and for both trained and untrained eye, an index of retention of improvement in VA after six months. The retention index ($R_I$) was again defined as in Zhou et al. (2006):

$$R_I = 100\frac{VA_{follow-up} - VA_{pre-training}}{VA_{post-training} - VA_{pre-training}} \quad \text{Eq. 3}$$

An $R_I$ of 100% indicates a full retention of the VA improvement following perceptual training, while an $R_I$ lower or greater than 100% indicates loss or further improvement of VA after the post-training measurement. An $R_I$ of 0% indicates no retention of VA improvement. The $R_I$ calculated for the trained eye was 98% (SEM: 13.9%), whereas the $R_I$ calculated for the untrained eye was 116.5% (SEM: 34.02%). We then performed a paired-sample t-test reporting that the $R_I$ for trained eye and the $R_I$ for the untrained eye were not significantly different ($t_9$ = -0.625, $p$ = 0.55), suggesting similar retention of perceptual training effects in both eyes. Moreover, $R_I$s calculated for the trained and untrained eyes were not significantly different from 100% ($t_9$ = -0.145, $p$ = 0.89, $t_9$ = 0.49, $p$ = 0.64 for trained and untrained eye, respectively (critical $p$ = 0.025), suggesting an almost full retention of the VA improvement after perceptual training.

## 3.4. Discussion

The present work investigated if a short (8 sessions) monocular behavioural training consisting of a visual contrast detection task using the lateral masking paradigm combined with hf-tRNS was able to improve VA and CS in adults with amblyopia and if

tRNS was crucial for improving VA and CS in patients with amblyopia. One group of amblyopic participants underwent online hf-tRNS, whereas the other group performed the same perceptual training but with Sham stimulation.

For what concern CS, the results obtained indicated that this perceptual training was able to improve CS in amblyopic adults. Even though the improvements obtained was slightly lower than those reported in previous studies that used a similar training (Polat et al., 2004) here, the number of sessions was importantly reduced compared to previous studies. The training length is a fundamental requirement that might limit the dropouts. CS improved in both eyes and in both real and Sham stimulation conditions, and for all the spatial frequencies tested. Differently to what expected, hf-tRNS did not enhance the effect of the perceptual training on CS. However, the magnitude of CS increment appeared to be higher in the hf-tRNS group than in the Sham group and specific for the trained eye. Although we trained a single spatial frequency, we obtained an improvement for all the spatial frequencies tested, indicating that PL generalizes to untrained spatial frequencies, as found in previous studies where contrast detection was trained (Casco et al., 2014; Huang, Zhou, & Lu, 2008; Polat et al., 2004; Zhou et al., 2006).

Regarding VA, only the hf-tRNS group showed improvement both in the amblyopic eye and in the non-amblyopic eye (patched during treatment). Mean improvements were of 0.19 LogMAR for the amblyopic eye and 0.11 LogMAR for the non-amblyopic eye. The absence of any significant difference between pre- and post-training in the Sham group might suggest that hf-tRNS was responsible for the transfer of PL to untrained visual functions precisely VA.

Moreover, we found that the VA improvement of almost 2 LogMAR in the hf-tRNS group was maintained six months after the training (follow-up). Therefore, the combination of training with a lateral masking paradigm (Polat et al., 2004; Polat and Sagi, 1993) and concurrent hf-tRNS showed a long-lasting effect on VA. This improvement is likely to reflect long-term neural plasticity in the amblyopic visual system. Other studies have given evidence for this plasticity in adults with amblyopia and have demonstrated how a CS training can transfer to other related untrained tasks such as VA (Campana et al., 2014; Chung, Li and Levi, 2006, 2008; Huang et al., 2008; Levi, Polat and Hu, 1997; Levi, 2005; Levi and Polat, 1996; Li et al., 2005; Polat, 2009; Polat et al., 2004; Zhou et al., 2006). For instance, Huang and colleagues (Huang et al., 2008) found that training in a grating detection task at their cut-off spatial frequencies improved not just CS at the trained frequency but also improved VA in the trained amblyopic eyes, and CS in the untrained fellow eye. Similarly, Polat and colleagues (2004) showed that VA improved after training in a very different and more basic task (contrast detection). The improvement in a higher-level type of task (VA) might depend on the improved quality of the low-level visual representation due to the practised stimulus-specific for early visual process.

These results indicated that the inaccurate pattern of visuospatial functions, typically observed in amblyopia, can be improved through PL. The improvements achieved was likely due to the long-term neural plasticity of V1, which is maintained even in an adult visual system (Gilbert et al., 2009).

It can be hypothesised that CS improvements are due to a weakening of lateral inhibitory interactions between V1 neurons tuned to specific orientations and spatial

frequencies (Polat, 1999; Polat et al., 2004), with a consequent decrease of interocular suppression (Harrad, 1996). Another possible mechanism is the strengthening of interactions between binocular neurons of the visual cortex that are suppressed in the amblyopia (Hess, Mansouri, & Thompson, 2011).

The transfer of improvement to a different task (VA) suggests that the benefits of the training on early vision processing may reflect hierarchically higher visual processing levels, which in turn depend on the quality of the lower-level visual representations (Polat et al., 2004).

Several studies on treatment practices of visual defects in amblyopia reported an improvement in visual functioning through the administration of PL (for a review see Levi et al., 2009); dichoptic training (Hess et al., 2012; Li et al., 2013; To et al., 2011) and video gaming (Achtman, Green, & Bavelier, 2008).

It has been shown that stimulating the occipital pole of healthy subjects with hf-tRNS combined with PL improved orientation discrimination and the improvement observed with hf-tRNS was higher than that reported when PL was combined with other electrical stimulation techniques (e.g., low-frequency tRNS, anodal-tDCS, cathodal-tDCS) (Fertonani et al., 2011).

Research is moving towards a combined approach in the rehabilitation of visual defects using NIBS, in order to boost neural visual plasticity and enhance the effects of existing behavioural regimes (Romei, Thut, & Silvanto, 2016; Spiegel et al., 2013a; Thompson, Mansouri, Koski, & Hess, 2008).

For example, a-tDCS has been successfully used in combination with Vision Restoration Therapy (VRT) in a patient with hemianopia to increase the suboptimal level of activity

of neurons in the damaged visual cortex (Plow et al., 2011). Moreover, a recent study in which that visual training was coupled with brain stimulation, revealed that tRNS but not a-tDCS, not only reduced the training period from months to weeks, but also led to fast improvement in patients with cortical blindness (Herpich et al., 2019).

In the present study, hf-tRNS could have increased the activity of inhibited monocular neurons tuned to specific orientations and spatial frequencies. However, the mechanisms through which hf-tRNS promotes neural plasticity at the level of the visual cortex are still debated. One underlying mechanism might involve the strengthening of weak connections through Hebbian learning, resulting in a recovery of function implicating various lateral, feedforward and feedback mechanisms (e.g. Li & Levi, 2004; Polat et al., 2004; Rosa, Silva, Ferreira, Murta, & Castelo-Branco, 2013). It has also been hypothesised that online hf-tRNS could induce a temporal summation of weak depolarising currents. This would induce a synaptic enhancement of neurons in the striate cortex and a consequent facilitation of the perceptual task (Fertonani et al., 2011; Terney et al., 2008). Therefore, tRNS, which repeatedly stimulates cortical neurons under their response threshold, could prevent the homeostasis of the system and could potentially strengthen the neural activity dependent on the specific task performed. According to recent models, facilitatory versus inhibitory effects of brain stimulation would depend on both the initial level of neural activation and the intensity of the stimulation. A weak stimulation, like that induced by hf-tRNS, could increase the firing rate of the most active neurons because tuned to the target stimulus (Miniussi & Ruzzoli, 2013; Silvanto & Cattaneo, 2017). According to the stochastic resonance (SR) theory, the neural noise induced by hf-RNS might have

enhanced the weak response of neurons receiving input from the amblyopic eye during training. SR explains how the addition of random activity (or noise) can enhance the detection of a weak signal. An optimal amount of noise would result in a maximum enhancement; on the other hand, further increasing the noise intensity would degrade the detectability or information content of a specific stimulus. Therefore, if a particular stimulus is below the detection threshold and never crosses it, such a stimulus is undetectable. This might occur because the stimulus is weak and/or because the neurons tuned to the stimulus are scarcely excitable. In the present case, both conditions occurred: stimuli were at threshold level and patients were trained with their amblyopic eye. However, when noise is added to a weak signal, threshold crossing may occur with greater probability (Miniussi, Harris, & Ruzzoli, 2013b; Schwarzkopf, Silvanto, & Rees, 2011; Van der Groen & Wenderoth, 2016; Ward, Doesburg, Kitajo, MacLean, & Roggeveen, 2006). The random activity introduced by hf-tRNS may also increase the synchronisation of neural firing through the amplification of (subthreshold) oscillatory activity of neurons (Ward, 2009) in the striate and extrastriate areas (e.g., V2) involved in contrast detection during PL. As a consequence, neural synchronisation may activate more neurons responding to the target, thus increasing target detectability. This phenomenon is known as neural synchronisation mediated by stochastic resonance (Ward, 2009).

It can also be hypothesised that the administration of 8 sessions of hf-tRNS alone is able to improve VA by enhancing the neural processing of information. In line with this explanation it is unclear why this benefit occurs just in VA, a task not trained, and it does not occur for CS that may take advantage from the training itself. On the other

hand, the strength of CS training itself could be powerful enough not to benefit from hf-tRNS. Although 8 sessions of hf-tRNS alone were not efficacious in improving VA in participants with mild myopia (whereas hf-tRNS combined with a contrast detection training were effective: Camilleri et al., 2016), it would be interesting to investigate the effects of 8 sessions of hf-tRNS in adults with amblyopia in a future study. We predict that hf-tRNS, when not coupled with a visual training able to differentially stimulate inhibited neurons of the amblyopic eye (concerning those responding to the fellow eye), would not produce any effect.

In summary, this study, according to the majority of studies cited, pointed to the existence of plasticity in adult human visual cortex after a treatment, specifically in response to a short perceptual training regime with a lateral masking paradigm combined with hf-tRNS.

Although hf-tRNS has been shown to promote the transfer of PL to untrained visual functions, precisely VA, more studies are necessary to fully understand the efficacy of hf-tRNS in boosting the PL and promoting generalisation to other visual tasks in patients with amblyopia.

In conclusion, several forms of plasticity remain efficient in the adult visual system, as PL showed. Understanding how these mechanisms work could open the way to innovative methods of diagnosis and treatment of many ophthalmic disorders challenging to treat.

## 3.5. Acknowledgements

A version of this chapter has been published in the Journal: *Neuropsychologia* and has been accordingly cited in the reference section: Moret, B., Camilleri, R., Pavan, A., Giudice, G. L., Veronese, A., Rizzo, R., & Campana, G. (2018). Differential effects of high-frequency transcranial random noise stimulation (hf-tRNS) on contrast sensitivity and visual acuity when combined with a short perceptual training in adults with amblyopia. *Neuropsychologia*, *114*, 125-133.

# CHAPTER 4. BOOSTING COGNITION USING EXERGAME AND TRANSCRANIAL RANDOM NOISE STIMULATION

## 4.1. Introduction

During the last 40 years, the use of video game has tremendously increased. Recently, specific games called “brain games” captured the interest of many researchers for their potential beneficial effects on cognition and behaviour (Green & Seitz, 2015; Palaus, Marron, Viejo-Sobera, & Redolar-Ripoll, 2017), inducing brain structural changes (Momi et al., 2018, 2019). Brain games have many advantages: they are stimulating, fun, cheap, easily available, user-friendly, and they provide immediate feedback about the performance. In addition to being enjoyable, they could include complex cognitive demands; recent studies have found that training based on brain games, which do not necessarily require physical exercise, can significantly improve abilities in cognition and perception (Achtman, Green, & Bavelier, 2008; Green & Bavelier, 2008; Maillot, Perrot, & Hartley, 2012).

In recent time, a particular type of video game called “exergame” has been developed. Exergame is a compound word formed by “exer”, that refers to exercise, and “game”, like gaming. It requires the player to interact using the body to perform the activity. Wii, Playstation, Xbox are all game consoles that offer different possibilities to perform several types of exergame.

The advantages deriving from the exergame are not merely due to the benefits associated with physical activity (Siegel, Haddock, Dubois, & Wilkin, 2009) and cognitive tasks (Best, 2013; Green & Bavelier, 2003) but involve an additional value

represented by the combination of them (Eggenberger, Theill, Holenstein, Schumacher, & de Bruin, 2015). A study in adults reported a moderate level of physical activity increased short-term plasticity in visual cortex (Lunghi & Sale, 2015) and improved cognition in elderly (Hughes, Seymour, Campbell, Whitelaw, & Bazzarre, 2009).

The exergames are also associated with high levels of appreciation and compliance (Maillot et al., 2012).

A recent review article highlighted the growing use of exergame for rehabilitation, for instance, in people with multiple sclerosis (Taylor & Griffin, 2015). De Giglio et al., (2015), used "Dr Kawashima's Brain Training" (DKBT; Nintendo, Kyoto, Japan) as a cognitive training with MS patients. Results showed improvements in information processing speed, executive functions and some aspects of quality of life (De Giglio et al., 2015). Interesting results have previously been found, with the same video game, in healthy young adults obtaining an improvement in executive functions, working memory, and processing speed (Nouchi et al., 2013), and also in elderly a beneficial effect has been found for cognitive functions (Nouchi et al., 2012).

Other recent studies investigated the effects on several tasks involving perception, cognition (Momi et al., 2018), and motor skills after action video game experience, showing that the most significantly enhanced effects were in the tasks strictly related to the functions trained (Green & Bavelier, 2008).

Furthermore, two neuroimaging studies showed consistent finding in structural modifications of the brain related to video game playing. Changes not only in the behavioural outcome but also of the brain were showed: after thirty hours of action

game training, structural brain changes associated with perceptual processes and attention were found, with long-lasting cortical thickness modifications up to 3 months (Momi et al., 2018). Similarly, Kühn and colleagues (2014), found a positive correlation between cortical thickness, precisely left-DLPFC and left-FEFs and video game training length (Kühn et al., 2014).

We chose the exergame, "Dr Kawashima's Body and Brain Exercises", that involves both cognitive functions, such as executive functions, processing speed and working memory, as well as motor functions. We utilised Xbox console through the Kinect device that has depth sensors cameras, which reproduce the body image and the actions on the screen without the requirement of any handheld controller or balance board (Figure 4.1). This exergame is aspecific, and it requires the player to execute multiple complex cognitive tasks, it is based on an adaptive procedure, so the difficulty of each exercise varies according to the level of performance of the participant. Consequently, the person is always pushed to work to the maximum of its potential, increasing his motivation and the possibility of obtaining an improvement. Likewise, the task is never too difficult for the participant, avoiding excessive frustration. It provides visual and auditory feedback, which work as powerful motivators too.

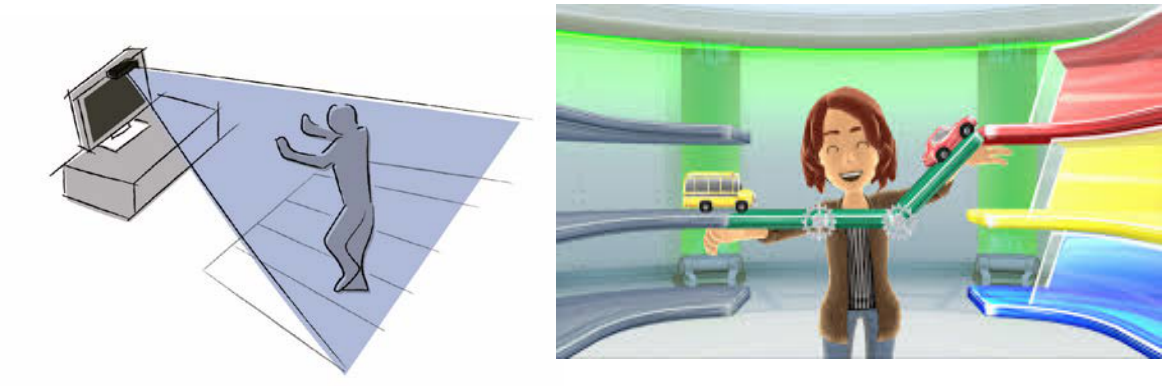

Figure 4.1. On the left, a schematic representation of a person playing an exergame; the small panel, positioned in front of the player, is equipped with depth sensor camera which detects and reproduces body movements on the screen. On the right, a representation of the participant avatar during an activity performance.

The phenomenon of enhancement of the performance of the untrained tasks is known as “transfer effect” (Boot, Blakely, & Simons, 2011; Boot, Kramer, Simons, Fabiani, & Gratton, 2008; Green & Bavelier, 2003; Miller & Robertson, 2011; Nouchi et al., 2012, 2013) and it is related to the brain plasticity mechanism (see paragraph 1.1).

So far, there is a lack of a systematic procedure to measure the effects and the transfer effect of video game training, and this may also be due to the several types of videogame used (Green & Bavelier, 2008). For instance, in older adults, in some case the transfer of game training was no significant (Ackerman, Kanfer, & Calderwood, 2010) but in others exergaming induced benefits on executive control, processing speed (Basak, Boot, Voss, & Kramer, 2008; Maillot et al., 2012), and memory span (McDougall & House, 2012); all results consistent with the previously reported advantages due to sedentary videogame training.

We tested Simple Reaction Time task and a Go/NoGo task with the 80% frequency of Go-trials (Go) which emphasize a prepotent tendency to respond and consequently to inhibit the action when a NoGo-trial (NoGo) appears (Wessel, 2018; Wright, Lipszyc, Dupuis, Thayapararajah, & Schachar, 2014).

We focused our interest on how the exergame training combined with hf-tRNS may affect executive control, behavioural inhibition and processing speed which, as stated in Salthouse (1996), refers to how quickly different types of processing operations can be carried out (Salthouse, 1996).

The so-called executive functions (EF), cognitive control, planning, execution, monitoring and inhibition, are always associated with the prefrontal cortex activation (PFC) (Hoshi, 2006; Mazzucchi, 2012; Miller & Cohen, 2001; Miller, Freedman, & Wallis, 2002; Wessel, 2018).

The PFC can be differently divided depending on the criteria: anatomical, functional or evolutional. Here, we focused on the PFC's functional properties which determine its subdivisions into three main regions: lateral, medial, ventral or orbital. The lateral face can be split itself into two parts: the ventrolateral prefrontal cortex (VLPFC) positioned in the inferior frontal gyrus (IFG) and the dorsolateral prefrontal cortex (DLPFC), located in the middle frontal gyrus, that represent our stimulation target area. Anatomically, the DLPFC includes Brodmann's area 8, 9, 46 (BA 8, BA 9, BA 46) and 10 (BA 10) (Brodmann, 1909; Cieslik et al., 2013) (Figure 4.2).

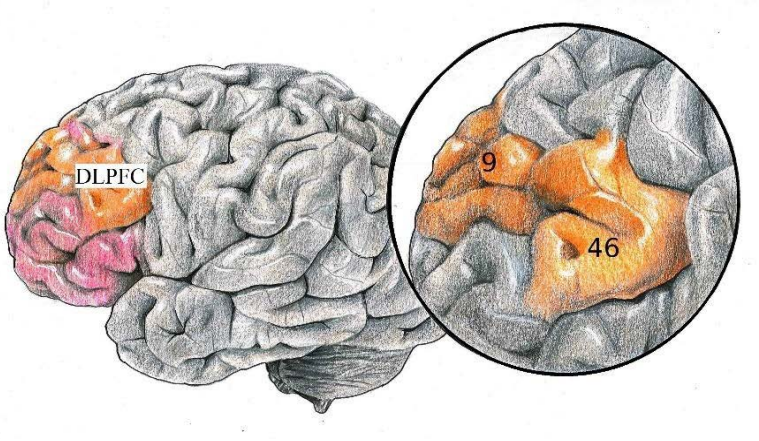


Figure 4.2. Sagittal representation of the left hemisphere: in pink the left prefrontal cortex, in orange the left dorsolateral prefrontal cortex (left-DLPFC), which includes the areas 9 and 46 of Broadman.

The DLPFC has many extrinsic and intrinsic connections with several regions such as the supplementary motor area (SMA), the pre-supplementary motor area (pre-SMA) (Picard & Strick, 2001), the frontal eye field (FEF) and to the posterior parietal associative cortices (Figure 4.3); most of these connections are reciprocal and may explain its top-down regulation role on information processing (Koechlin, Ody, & Kouneiher, 2003; MacDonald, Cohen, Stenger, & Carter, 2000).

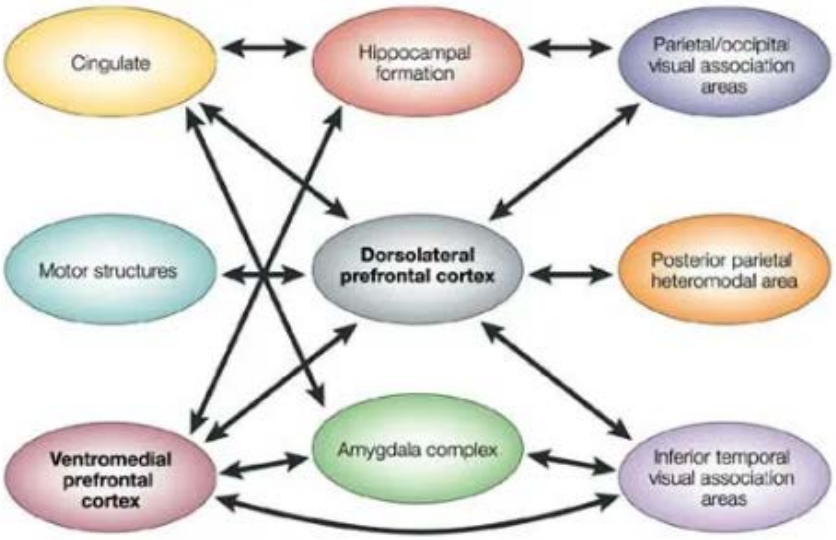


Figure 4.3. Schematic representation of the connectivity between the left dorsolateral prefrontal cortex (left-DLPFC), and other brain regions (adapted from Wood & Grafman, 2003).

Many studies marked the DLPFC for its involvement in cognitive control processes such as motor planning, organization, regulation (Grier, 2005) and control inhibition (Wessel, 2018); the connection with the pre-SMA is particularly relevant when a suitable response or the inhibition of an inappropriate one is requested (Simmonds, Pekar, & Mostofsky, 2008); to investigate this process the Go/NoGo task seems the case in point.

Brain plasticity is the basis of learning, and it depends on the connections among neural populations. Non-invasive brain stimulation may be one way to contribute to the cortical changes to boost the learning rate. We know that transcranial electrical stimulation can modulate neural activity and modify the connection strength among neurons (Fertonani et al., 2011; Mulquiney, Hoy, Daskalakis, & Fitzgerald, 2011; Snowball et al., 2013; Terney et al., 2008).

In the present research, we investigate the effect of hf-tRNS, and we combined it with the exergame training, to understand any potential individual effect and/or in terms of enhancement due to synergistic effects caused by its interaction with the training. Multiple studies used transcranial stimulation to investigate the inhibitory control through the Go/NoGo task, all targeting DLPFC using different montages. A recent review by Brevet-Aeby and colleagues (2016) offered an extensive overview about noninvasive brain stimulation techniques to investigate the relationship between the PFC and impulsivity, strictly related to executive control (Brevet-Aeby, Brunelin, Iceta, Padovan, & Poulet, 2016).

So far, many of the studies about DLPFC stimulation regarded mainly tDCS protocol.

A study that considered the effect of the time of the stimulation, showed that 10 minutes of tDCS on DLPFC bilaterally during an air traffic control task, was able to induce the participants to identify more targets when the anode was placed on the left-DLPFC, and the stimulation was delivered at the beginning of the task, during the first 10 minutes. False Alarms (FA) diminished in both left- or right-DLPFC targets, with the stimulation delivered the last 10 minutes (Nelson, McKinley, Golob, & Warm, 2014).

Beeli et al., (2008) reported a differential effect between anodal- and cathodal-stimulation in the number of FA with the cathode placed over the right-DLPFC and the anode on the ipsilateral mastoid. A significant increase of the FA in a Go/NoGo task were obtained with the cathodal stimulation while no changes were found for the anodal- or Sham stimulations (Beeli, Casutt, Baumgartner, & Jäncke, 2008). By contrast, Boggio and colleagues (2007) compared a-tDCS over the left-DLPFC with Sham and occipital stimulations in patients with major depressive disorder, demonstrating an increase of the accuracy tested with an affective Go/NoGo task (Boggio et al., 2007). This result supported the involvement of left-DLPFC mood modulation (Mayberg, 2003).

After a-tDCS over the left-DLPFC, decreased reaction times (RTs) in the Sternberg test were obtained but only in high-interference condition, compared to Sham stimulation (Gladwin, den Uyl, Fregni, & Wiers, 2012). Another study concentrated on the contribution of the genetic factor, investigated the effect of c-tDCS applied on left-DLPFC and the anode over the right supraorbital region, finding a response inhibition decrease in a Go/No Go task, only in individuals homozygous for the Val-

allele of the COMT Val (108/158) Met polymorphism. They suggested that the effect of the tDCS stimulation may be modulated by genetic factors (Nieratschker, Kiefer, Giel, Krüger, & Plewnia, 2015).

In contrast, in a study investigating the potential improvement of inhibitory control in adolescents with ADHD symptoms, only when left-DLPFC was stimulated with c-tDCS, an increase of the inhibition accuracy was found in comparison with Sham stimulation(Soltaninejad & Nejati, 2015).

Newly, Brevet Aeby et al., (2019) investigated the effect of tRNS with 1 mA offset, comparing 20 minutes of three consecutive sessions, separated by 30 minutes, of active tRNS (3A), with 1 active and 2 Sham (1A2S) and 3 Sham (3S) tRNS conditions. The target area was the bilateral-DLPFC with the anode (referred to 1 mA offset) over the left-DLPFC. The aim was to investigate inhibitory control in healthy subjects. They found a decrease of RTs in the Go-trials, just in 3A condition as compared to Sham stimulation (Brevet-Aeby et al., 2019). This result indicates that tRNS was able to boost the response execution after 3 consecutive active sessions, with no effect of a single session (1A2S).

Although the literature on tRNS effects is still scarce (tDCS is still the most investigated protocol), in this study we used hf-tRNS, which in addition to being the most recent technique, it has shown encouraging results mostly using high-frequency band.

Several studies in the literature have demonstrated the efficacy of hf-tRNS when combined with perceptual training (Campana et al., 2014; Fertonani et al., 2011; Moret et al., 2018). Fertonani and colleagues (2011), stimulating with hf-tRNS the

occipital cortex, obtained an increase in performance in a perceptual learning task (Fertonani et al., 2011). In the same way, the stimulation of the visual cortex with hf-tRNS combined with perceptual training was effective in improving visual functions, specifically visual acuity and contrast sensitivity in people with mild myopia (Camilleri et al., 2014). Similarly, Moret and colleagues (2018; see also Campana et al., 2014) have shown in adults with amblyopia, that 8-sessions of hf-tRNS applied over V1, combined with a contrast sensitivity training, was able to lead to significant improvements not only in contrast sensitivity but also in visual acuity, a visual function not directly trained (see Chapter 3).

There are only a few studies that combined cognitive training (not necessarily using exergame) with tRNS on the DLPFC (Santarnecchi et al., 2015).

Prichard et al., (2014) investigated different stimulation protocols specifically tDCS and tRNS, on M1 unilaterally and bilaterally, demonstrating a different time interaction with a motor training on a tracing task over three consecutive days and providing their more beneficial effects in enhancing motor skill learning compared to Sham stimulation (Prichard et al., 2014).

Another study, in which 5 days of cognitive training were coupled with tRNS applied on the bilateral-DLPFC, showed a behavioural improvement in speed of calculation and memory-recall-based arithmetic learning, demonstrating a long-term enhancement associated with hemodynamic responses specifically within the left-DLPFC (Snowball et al., 2013).

Brem et al., (2018) investigated the possibility of a transfer effect, comparing four stimulation protocols tRNS, tDCS, multifocal tDCS and multifocal tACS combined with

9-sessions of 30 minutes of gamified tasks on executive functions including working memory, inhibition, and cognitive flexibility training. All the protocols, apart from the multifocal tACS, showed far transfer effects to fluid intelligence (Gf) (Brem et al., 2018).

Cappelletti et al., (2013) coupled tRNS over distinct areas of the brain with intense cognitive training, demonstrating a long-term improvement in a trained numerosity discrimination task. Importantly, the best outcome was performed by the group trained with the stimulation targeting the parietal lobes, regions critical for quantifying processing. Additionally, they showed an improvement in time- and space-discrimination, cognitive skills not directly trained, indicating that a generalised transfer occurred (Cappelletti et al., 2013).

TRNS seems to represent a promising technique capable of enhancing the effects of cognitive training, accelerating the benefits and promoting transfer effects of learning, even to skills not strictly related to those being trained (far transfer effects).

In light of the above favourable results, for the first time, we applied hf-tRNS, ranging from 100 to 640 Hz, over the left-DLPFC and V1 in the healthy subject, to evaluate the effect of this stimulation protocol in combination with an exergame training.

Precisely, the aims were to evaluate the effectiveness of the exergame, “Dr Kawashima’s: Body and Brain Exercises”, and the potential effect of the stimulation protocol chosen. Moreover, we were interested in investing any benefit of tRNS when associated with this exergame in improving executive control response, precisely the motor and cognitive processing speed and the inhibitory response.

We expected an improvement in RTs in both Simple Reaction Time task and Go/NoGo task and a reduction in FA, especially in the stimulation conditions. Moreover, since tRNS seems to boost the effect in conjunction with training, we expected a better performance in the group who received both training and real stimulation.

## 4.2. Methods

Participants

Forty-nine young healthy participants (12 male, mean age 24 years) took part in this study and gave written informed consent according to the Declaration of Helsinki. This study was approved by the local Ethics Committee (Protocol Number: 2397).

All participants were assessed for stimulation contraindications (Rossi et al., 2009) through a specific questionnaire and, since the experimental design involved the use of the entire body to perform the training, motor difficulties were considered an exclusion criterion.

Participants were randomly assigned to one of the four conditions: Active stimulation + Training (A_T), Sham stimulation + Training (S_T), Stimulation with No Training (A_NT), and Control (C) (Table 4.1).

| Group | N | Gender | | Age | | Education | |
|---|---|---|---|---|---|---|---|
| | | F | M | Mean | SD | Mean | SD |
| **S_T** | 12 | 8 | 4 | 23,8 | 4,0 | 17,8 | 2,7 |
| **A_T** | 12 | 8 | 4 | 23,8 | 4,5 | 16,6 | 2,3 |
| **A_NT** | 12 | 8 | 4 | 25,6 | 7,3 | 17,3 | 1,9 |
| **C** | 13 | 9 | 4 | 21,1 | 2,2 | 15,1 | 2,0 |

Table 4.1: Characteristics of the participants for each condition.

M: males; F: females; M: male; SD: standard deviation

Apparatus

In order to perform the assessment tasks, stimuli were generated, and responses were collected, using Psychotoolbox 3.0 and Matlab R2014a (v.8.3) running on a PC, and showed on a hp p1230 screen with 1280 x 1024 resolution. Manual responses were given by pressing the spacebar on a standard computer keyboard. Viewing distance was 57 cm.

The laboratory was also equipped with an Xbox 360 and a Kinect device that has a depth sensor camera. Cognitive exercises of the exergame “Dr Kawashima’s Body and Brain Exercises" were carried out using a 40’’ liquid-crystal display (Samsung Model UE40K5510AKXZT) with a 1920 x 1980 resolution. Viewing distance was approximately 2 m.

For transcranial random noise stimulation (tRNS), a battery-driven stimulator (BrainSTIM, EMS) and two electrodes, 4x4 cm size, and a non-elastic bandage to hold them were used. The stimulator was placed inside a pouch tied at the waist and placed on the back of the participant.

Assessment

The participants were required to perform two tasks: Simple Reaction Time (sRT) task and Go/No-Go task.

sRT task consisted in pressing as quickly as possible the keyboard spacebar whenever a blue rectangle (2.5 x 5 deg; R0 G0 B255) appeared in the centre of a grey screen (R128 G128 B128). In order to help the participant keeping the attention to the right position, a white fixation cross appeared for 200 ms immediately before the target; the inter-stimulus interval (ISI) was set as 0.8 s, plus a random interval ranging from

0.0 s to 0.8 s in order to avoid automatic responses. The stimulus timeout was set at 2 s. During sRT task, 40 consecutive trials were presented.

Go-NoGo task had the same parameters but consisted of two stimuli: a blue rectangle, the Go stimulus, which required the subject to respond as quickly as possible, and a red rectangle (R255 G0 B0), the NoGo stimulus; in this case, the participant was asked to inhibit the motor action. The total trials were 50 stimuli, 40 Go trials (80%) and 10 NoGo trials (20%), to evoke a prepotent motor response, and consequently an inhibitory control requirement (Wessel, 2018).

Transcranial random noise stimulation

The stimulation protocol was set at high-frequency (100 - 640Hz) random noise for 25 minutes with a current intensity of 1.5 mA and 0 mA offset. Current linearly increased in intensity up to 1.5 mA during the first 30 s of stimulation. The current density was 0.09 $mA/cm^2$, within the safety limits (i.e., below 0.1 $mA/cm^2$) (Poreisz et al., 2007). In the Sham condition, the current linearly increased for the first 30 s up to a 1.5 mA and then decreased to 0 mA the next 30 s.

The current was delivered using a pair of rubber electrodes covered by sponges soaked in saline solution. The electrodes were 16 $cm^2$ large, positioned with the centre above the left-DLPFC and the V1 both localised according to the 10-20 EEG-system and marked with a special pencil on the scalp.

Exergame training

The exergame “Dr Kawashima’s Body and Brain Exercises”, could be classified as a sensory-cognitive-motor training consisting of an interactive video game-based

cognitive exercise. The Kinect sensors detect position and timing information that is then used to provide participants with real-time visual feedback. This exergame includes 20 unique games, ranging from math, logic, reflex and memory, all physical-related exercises using the full-motion capabilities. Each activity focuses on specific cognitive function such as working memory, RT, processing speed and executive functions, besides requiring motor planning and execution. Each game included three difficulty levels , and in each level the difficulty grows depending on the participant's performance, to ensure adaptation and progression to the abilities of each player. Also, the players can track their daily progress.

One of the most crucial features of this exergame is the time available to perform a task: to complete a task the maximum time available is fixed, and sooner an activity is performed, higher is the result obtained, and consequently, the level reached. This was thought to induce the participant to become more efficient in less time.

Ten activities have been chosen in order to train processing speed, RT and inhibition, besides requiring motor planning and attention. The activities were named numerical balloons, coloured balloons, traffic policeman, turbulent mice, turn and discover, memory step, golden ball, what time is it, radar and perfect couple. The order of the activities was maintained identical for all participants and all sessions training.

Experimental procedure

The study was performed at the Department of General Psychology at the University of Padova. The experiment was explained to the participants, and they all gave written informed consent.

In the initial screening phase, exclusion criteria through a questionnaire and the absence of physical difficulties were verified. Participants were then randomized in 4 groups. Two groups received hf-tRNS or Sham stimulation on the left-DLPFc and V1, and were trained with ten activities of the exergame "Dr. Kawashima's Body and Brain Exercises". A third group underwent to hf-tRNS with no training, the control group performed only the assessment at Time 0 (T0) and Time 1 (T1) with the same time interval of the other groups. The assessment consisted of two tasks: Simple Reaction Time task and Go/No-Go task. Each task started with 10 practise trials in order to familiarise with the task.

The adaptive exergame training was carried out in 8-sessions of 45 minutes each, with hf-tRNS applied during the first 25 minutes. The total protocol lasted 2/3 weeks, for a total of 10 sessions, consisting of 8-training sessions plus 2 sessions of pre- (T0) and post-training (T1) evaluations (Figure 4.4). For each participant, the same researcher conducted the assessment at T0 and T1, and a different researcher conducted the training procedure.

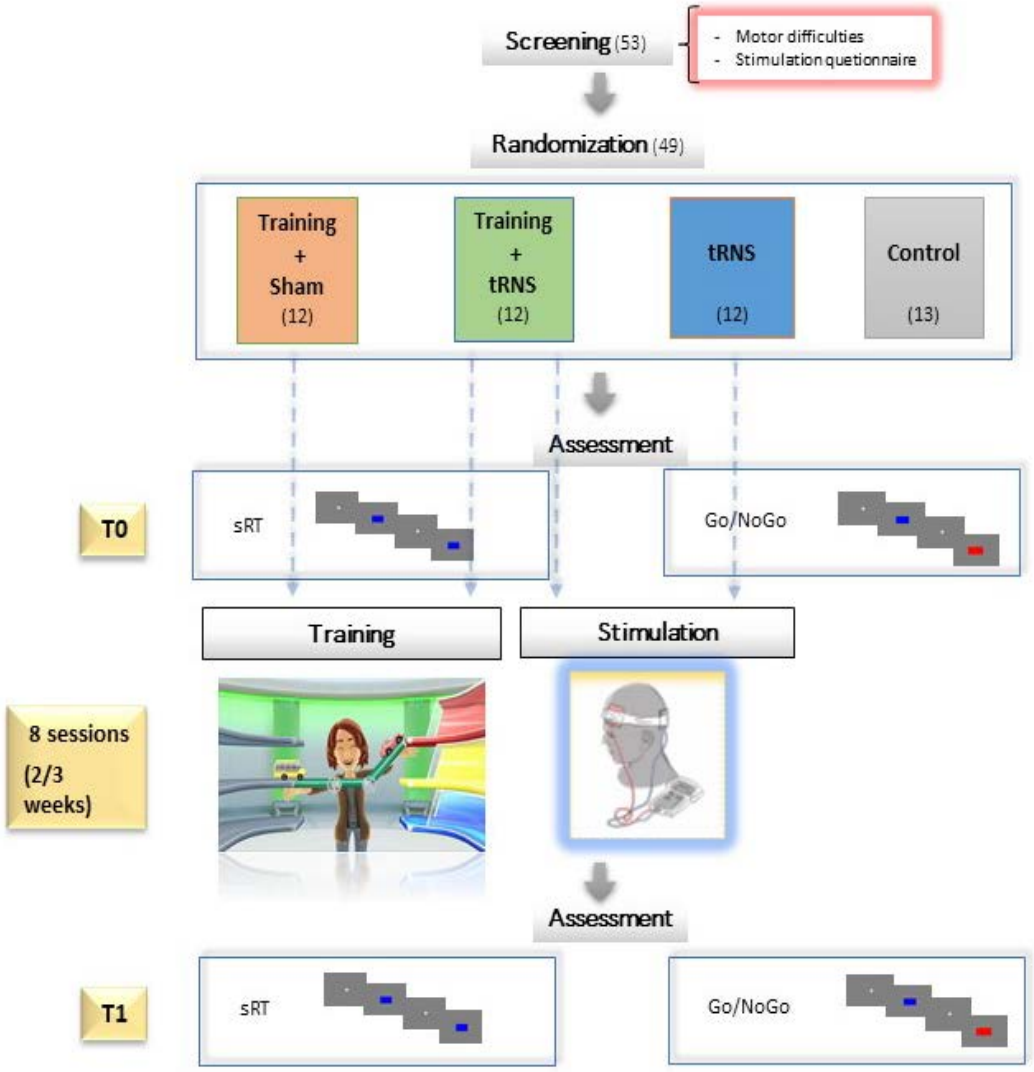


Figure 4.4. Flowchart representing the experimental design

Data Analysis

The median of reaction times (RTs) has been calculated for each participant. For the Go/NoGo task, besides the median RTs for Go trials, the number of correct responses related to Go trials (HITs) and the number of incorrect responses of No-Go trials (False Alarms - FAs) had also been collected (Table 4.2).

| | sRT | | Go/NoGo | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | T0 | T1 | T0 | | | | T1 | | | |
| | RT | RT | Go_RT | %HITs | % FA | d' | Go_RT | %HITs | % FA | d' |
| S_T | 238 (18) | 229 (14) | 290 (26) | 99,6 | 8,3 | 5,7 | 287 (21) | 99,8 | 6,7 | 6,2 |
| A_T | 253 (42) | 237 (25) | 322 (31) | 98,3 | 5,8 | 5,5 | 297 (18) | 99,0 | 3,3 | 5,8 |
| A_NT | 241 (26) | 245 (17) | 312 (27) | 97,5 | 4,2 | 5,3 | 292 (19) | 98,8 | 7,5 | 4,7 |
| C | 231 (13) | 229 (17) | 305 (33) | 98,7 | 4,6 | 5,3 | 303 (23) | 98,5 | 7,7 | 5,3 |

Table 4.2. Mean and standard deviation of reaction times of sRT task and Go/NoGo task for each experimental condition expressed in milliseconds. A_T: Active stimulation + Training; S_T: Sham stimulation + Training; A_NT: Stimulation with No Training; C: Control; RT ms: reaction time in millisecond; T0: pre.-test; T1: post-test; HITs: correct responses; FA: False allarm; d':d prime

Furthermore, for the Go/No-Go task, another variable has been created for each participant to combine speed and accuracy, the so-called Inverse Efficiency Score (IES) (Townsend & Ashby, 1978) (Formula 1):

$$IES = \frac{reaction\ time\ (RT)}{proportion\ of\ correct\ response}\ ms$$

Where RT is the median Go-RT of a single participant and the proportion of correct responses is the accuracy of a single participant.

Given the high percentage of correct response( >97%) and considering that the task performance is represented by a balance between HITs and FA, we calculated d prime (d') which indicates the ability to monitor performance in order to adaptively balance demands to detect and to rapidly respond to appropriate stimuli (Go) with the need to inhibit responses to non-targets (No-Go). It, therefore, represents the most comprehensive measure of overall task performance (Wickens, 2002).

Finally, for both simple Reaction Time and Go/NoGo tasks, RT percentage change was calculated by subtracting the T0_score from the T1_score and dividing the result by the T0_scores and finally multiplying it by 100% to obtain the percentage of variation from the baseline and have a measure of the improvement after the treatment (Formula 2).

$$\%\, change = \frac{T1_{score} - T0_{score}}{T0_{score}}\ x\ 100\,\%$$

## 4.3 Results

*Simple Reaction Time (sRT)*

To evaluate any difference among groups at T0 we ran a two-ways ANOVA with sRT as dependent variable and Stimulation and Training as Between-Subject variables with Age as covariate. No significant effect has been found for Stimulation ($F_{1,44}$ = 3.527, ρ=0.07, $\eta^2_p$=0.07), Training ($F_{1,44}$ =2.289 , ρ=0.14, $\eta^2_p$ =0.05), nor for the interaction Stimulation x Training ($F_{1,44}$ = 0.08, ρ=0.78, $\eta^2_p$ =0.002).

To investigate any effect of the treatment, we ran a repeated-measure ANOVA with Time (T0, T1) as Within-Subjects and Stimulation and Training as Between-Subject variables. The results revealed no significant effect of Time ($F_{1,45}$ =2.76, ρ=0.103, $\eta^2_p$ =0.06); regarding the interactions, only Time x Training showed a significant effect ($F_{1,45}$ = 4.504, ρ=0.039, $\eta^2_p$ =0.91). To explore the interaction Time x Training we run paired sample t-tests. Post-hoc comparisons reported a significant improvement for just the groups who received the training t(23)=2.431, ρ=0.023.

Trained participants became, on average, 12ms faster; in percentage, the training led to about 4% increase in the performance compared to the baseline (Figure 4.5). Neither the interaction Time x Stimulation nor the interaction Time x Training x Stimulation were significant, indicating that Stimulation was not able to modulate the performance in any group.

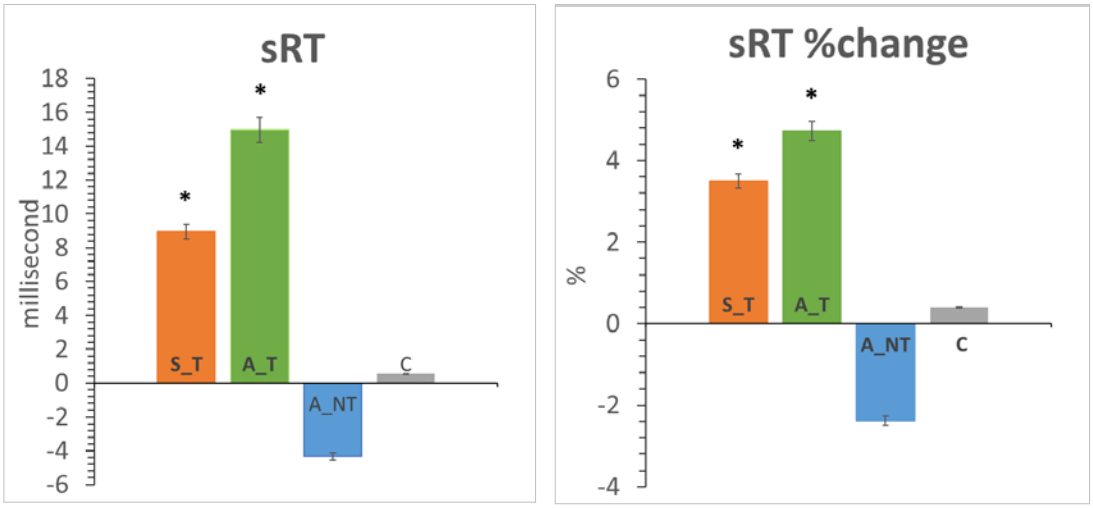


Figure 4.5. Simple reaction time task: on the left the mean of RT increase/decrease expressed in millisecond between T0 and T1 for each experimental condition; on the right the mean RTpercentage change between T0 and T1 for each experimental condition. A_T: Active stimulation + Training; S_T: Sham stimulation + Training; A_NT: Stimulation with No Training; C: Control.

*Go/No-Go task*

Similar analyses on the median-Go-RT (Go-RT) were conducted for Go/No-go data, adding the IES measure, which takes into account any change in the accuracy of the response.

To assess any difference among groups in Go-RT at the baseline, we ran a two-ways ANOVA with T0-Go-RT as dependent variable and Stimulation and Training as Between-Subject variables and Age as covariate. No significant effect has been found

for Stimulation ($F_{1,44}$ = 3.64, ρ=0.63, $\eta^2_p$=0.76), Training ($F_{1,43}$ = 0.61, ρ=0.44, $\eta^2_p$ =0.01), nor for the interaction Stimulation x Training ($F_{1,43}$ = 2.59, ρ=0.11, $\eta^2_p$ =0.06).

We conducted a Repeated Measure ANOVA with Time (T0 and T1) as Within-Subject and Stimulation and Training as Between-Subject variables. We obtained a significant result for the main effect Time ($F_{1,45}$= 10.803, ρ=0.002, $\eta^2$=0.194) and for the interaction Time x Stimulation ($F_{1,45}$ = 7.003, ρ=0.011, $\eta^2$=0.13). To further explore the interaction between Time and Stimulation, we compared T0 vs T1 measurements for stimulation condition with paired sample t-tests. Only the groups who received stimulation showed a significant improvement (t(23)=3.936, ρ=0.001). This result showed the effect of the stimulation in inducing a better performance with a mean improvement of 22 ms, about 7% better as compared to the initial outcome (Figure 4.6).

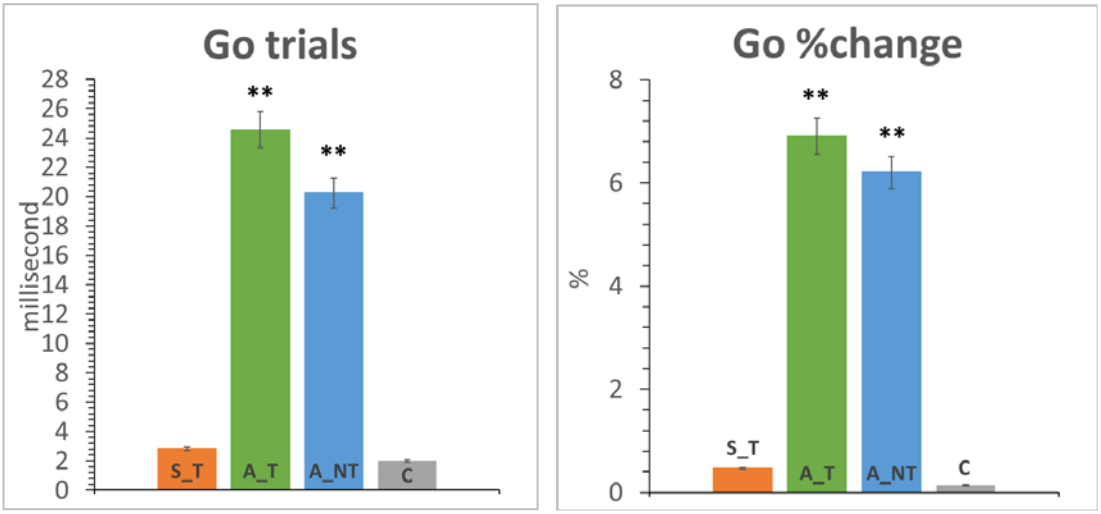


Figure 4.6. Go/NoGo task: on the left the mean Go-RT trials increase/decrease between T0 and T1 for each experimental condition; on the right the mean of the percentage change between T0 and T1 for each experimental condition. A_T: Active stimulation + Training; S_T: Sham stimulation + Training; A_NT: Stimulation with No Training; C: Control

We found comparable results for IES-scores, which also considered the accuracy; a Repeated Measure ANOVAs with Time (T0, T1) as Within-Subject and Stimulation and Training as Between-Subject revealed a significant effect of Time ($F_{1,45}$ = 9.79, ρ=0.003, $\eta^2$=0.18) and interaction Time x Stimulation ($F_{1,45}$ = 6.73, ρ=0.013, $\eta^2$=0.13). The paired sample t-test post-hoc comparisons revealed a significant improvement in the stimulation groups only t(23)=3.538, ρ=0.002, confirming that participants who received the stimulation improved Go-RT, and that this was not due to an RT-accuracy trade-off.

Even though the IES-scores considered the accuracy and the speed together in a single variable, in this study, we were interested in cognitive control, a process mostly related to the number of FA committed. Therefore, we calculated the difference (T1-T0) for both HITs and FAs to investigate any change in accuracy (HITs) and inhibition control (FAs) after the treatment. We performed a MANOVA with HITs and FAs as dependent variables and Stimulation and Training as Between-Subject. The results showed no effect of Stimulation ($F_{1,45}$ = 0.78, ρ=0.38, $\eta^2$=0.017) or Training ($F_{1,45}$ = 0.11, ρ=0.91, $\eta^2$=0.000) in HITs. No effect of Stimulation ($F_{1,45}$ = 0.006, ρ=0.94, $\eta^2$=0.000) or of Training ($F_{1,45}$ = 1.89, ρ=0.18, $\eta^2$=0.040) were found in FAs, indicating that all groups maintained the same pattern or response after the treatment. Moreover, although the d' scores obtained were high, to verify any change in the ability to distinguish between target and non-target we calculated a Repeated Measure ANOVA with Time (T0 and T1) as Within-Subject and Stimulation and Training as Between-Subject variables. No significant effect has been found for Time ($F_{1,45}$ = 0.005, ρ=0.946, $\eta^2_p$=0.001), nor for the interactions.

Summarising, the groups who received stimulation became faster in Go-RT, preserving the same accuracy and inhibition control in responding.

## 4.4. Discussion

The present study aimed at investigating the role of hf-tRNS on the left-DLPFC together with an exergame training in enhancing motor and cognitive performance in healthy subjects. We investigated whether 8-sessions of adaptive cognitive training and tRNS, either independently or combined, were able to boost processing speed and cognitive control.

We created an orthogonal design where the participants were randomly assigned to four groups. The first group, labelled S_T, performed 8-sessions exergame training with Sham stimulation; the second group A_T, performed the same training with active stimulation; the third group A_NT, received only the stimulation for 8 days with the same active protocol of the second group; and the fourth C, the control group, did not carry out any training nor was stimulated and did the assessment at T0 and T1, with the same time interval, about 2/3 weeks, as the other three groups.

The cognitive-motor training performed was expected to improve motor RTs in both simple Reaction Time and Go/NoGo tasks, due to its increasingly request of faster and more accurate responses.

Training involving speeded-exercise produce modifications in neural mechanisms, changes that also depend on the effort the training claims, as shown by neuroimaging studies (Takeuchi & Kawashima, 2012).

Even if this exergame was not created for research purposes, it has an adaptive procedure which we supposed may have led the participant to exercise at the maximum of his/her ability and, consequently, to improve the rate of learning. We theorised an improvement even in the Go/NoGo task due to the features of the exergame tasks selected since many activities involved executive control. For example, during “Coloured balloon “ (a type of Stroop task where the participant was asked to burst the balloon matching the colour word written in a different colour int) the ability to inhibit an automatic response, especially when a ballon coloured as the word int was present (interference), was requested. Also, in “Turbulent mice”, in where the player has so hit only a category of the mouse but not another one, similar to the Go/NoGo logic.

Moreover, since left-DLPFC is involved in the top-down regulation of cognitive control (MacDonald et al., 2000), we hypothesised that tRNS application would have increased this regulation resulting in an improvement in both sRT and Go/NoGo task. Specifically, in sRT due to the strong connections to the pre-SMA and SMA and the involvement of DLPFC in information processing (Koechlin et al., 2003); in Go/NoGo task because of the specificity of DLPFC which has been correlated with inhibitory response (Grier, 2005; Simmonds et al., 2008; Wessel, 2018).

Additionally, we assumed that online-tRNS application would have led to an increase in the effect of the training (Cappelletti et al., 2013; Pirulli et al., 2013). We hypothesised a better performance of the group who performed the exergame training with the active stimulation due to an increase in the cortical excitability of

the DLPFC already involved and prompted by the exergame activity, as a result of a synergetic effect.

We selected two tasks, which are sRT and Go/NoGo tasks to measure information processing speed, executive and inhibitory control.

Regarding sRT task, which consists of a measure of the response time required for pressing a bottom immediately after the appearance of the stimulus, the results showed improvement only in the groups that carried out the training, regardless whether a group received or not the stimulation.

Given the nature of the exergame which requires a considerable fast motor response, and therefore a continuous activation of the motor areas, the improvement obtained in the sRT task, likely due to the exergame practice, was expected. The exergame used, although it is aspecific, which means that it does not train a specific function, requires the participant to be always faster in responding in order to get a better score and level up. Furthermore, all the activities carried out required a cognitive effort to process the information. Therefore, we believe that there was an increase in the efficiency of the motor system and in information processing likely due to the greater communication activation between the brain areas involved. Improvements that can be translated in a shorter time to respond to a simple task like the sRT task.

What we showed is that 8-training sessions with this exergame allowed to gain about 12 ms in sRT, that is not too much but still an improvement. The present study was conducted with young people, but it would be interesting to study its potential benefits in the elderly since previous studies showed that older subjects accumulate sensory information as rapidly as young subjects, but they take more time in

responding (Ratcliff, Thapar, & McKoon, 2001). Woods et al. (2015) estimated that latency increases significantly with age 0.55 ms/year (Woods, Wyma, Yund, Herron, & Reed, 2015). It could be interesting if the RT gained after the training in young people could be reproducible with ageing people.

Concerning the Go/NoGo task, only the groups which received stimulation showed an improvement such as a reduction of the RTs of Go-trials with no improved performance when training and stimulation were combined. The fact that the stimulation induced a reduction of the RTs in the Go-trials but not in sRT task, indicate the specificity of the stimulation for the former task. Go/NoGo is a higher-level demanding task compared to sRT task. It consists of a set of semi-independent processes, including stimulus encoding, stimulus-response association, rate of information processing, speed-accuracy trade-offs, and motor response (Karalunas, Nigg, & Huang-Pollock, 2012), and also it involves inhibitory control, related to the activation of the frontal area (Simmonds et al., 2008). The stimulation could have played a fundamental role by enhancing the excitability of DLPFC and consequently, by improving the processes involved in this task, perhaps strengthening the processing speed, which is crucially involved in any motor and cognitive activity and it has been proposed as a key component for the brain functioning (Takeuchi & Kawashima, 2012).

We know that the DLPFC is part of a network which includes premotor and somatosensory areas and it is also thought to be the responsible for transforming the input sensory signals into distinctive output motor orders (Heekeren, Marrett, Bandettini, & Ungerleider, 2004; E. K. Miller & Cohen, 2001). This process may have

been reinforced by the tRNS, which may have contributed by inducing more neural activation, thus enhancing the excitability (Shafi, Westover, Fox, & Pascual-Leone, 2012). Moreover, as stated by Fertonani and colleagues, high-frequency ranges up to 1000 Hz could be ideal for interacting with neural communication (Fertonani et al., 2011) for the reason that the time constant of the cell body and dendrites is between 1 and 10 ms (Kandel, Schwartz, & Jessell, 2000), thus, preventing the neuronal membrane potential decay. It follows that a reduction of RT may be the result of the boosted network-communication.

Similarly to what we found with 8 sessions of tRNS, Aebey et al., (2018) showed that 3 consecutive active-tRNS sessions decreased RTs at the Go trials as compared to Sham.

The mechanism through which we hypothesised the tRNS had enhanced the response execution could be related to the possibility that the random noise current introduced into the brain may be able to alter the membrane resting potential. Operating on sodium and calcium channels, it causes larger sodium ions inflow and consequently prolonged depolarisation, resultant in an increase in the firing rate of the stimulated neurons (Antal, Terney, Poreisz, & Paulus, 2007; Fertonani et al., 2011; Schoen & Fromherz, 2008). This process can produce temporal summation on postsynaptic neurons which we hypothesise could likely be flanked by another process, the spatial summation. While the temporal summation consists of a quick succession of the firing of the same presynaptic neuron, the spatial summation takes place thanks to the contribution of more presynaptic axons that bring about

threshold simultaneously. Since tRNS is not focalised, it might elicit more activity-input from multiple presynaptic cells.

We found that participants who received 8 days of stimulation showed a decrease RT of about 27ms in a task where inhibition is required. What we have achieved is higher speed in a simple decision-making task that involves stimulus-response association and inhibitory control. The latter is more related to NoGo trials and what we found was a sort of floor effect at pre- and post-test, with a mean for each group less the 1 False Alarm, therefore without any opportunity to improve the performance. This result could be due to the task too easy or to the few numbers of NoGo trials and the high d' obtained in all groups is in favour of this hypothesis.

In any case, the 27 ms gained in Go-trials is worth considering the speed/accuracy trade-off remained constant as shown using the IES and also comparing the HITS and FA with no significant difference between the pre and post-test. The improvement in RTs and not in the accuracy is a result in line with what already found in healthy subject using a-tDCS (Dedoncker, Brunoni, Baeken, & Vanderhasselt, 2016).

The present study provides an excellent tolerance to 8-session tRNS of 25 minutes each: no participant reported side effects, and none was able to feel any cutaneous perception of the stimulation. This is a fundamental outcome considering the lack of safety records, especially for several sessions in combination with an exergame.

Even though the mechanism of action needs to be investigated in detail, in light of these results and those of the previously reported studies which show the benefit of tRNS, more research is necessary to use tRNS as a potential additional technique for clinical cognitive and motor enhancement. For instance, studies on inhibitory control

might be relevant for clinical populations such as people with ADHD or for substance use disorders (Coles, Kozak, & George, 2018), where it is known that impulsivity is one of the main symptoms (Winstanley, Eagle, & Robbins, 2006). Surely it is not possible to generalise the effect in a simple Go/NoGo task to the impulsive behaviour, but from this study, it seems that the tRNS if repeated, induces a better control of the executive action, so the processing of the information and in the reaction associated to it.

The number of trials of both tasks was kept low, 40 and 50 trials, to avoid any practice effect. The no significant difference found between pre- and post-test in the control group proved this aim. Nevertheless, the limited number of trials might be partially responsible for the unobserved effect on FA, a measure of a potential improvement in the inhibitory control.

The lack of the exergame effect in improving Go-RT was maybe due to the aspecific nature of the cognitive training performed, the insufficient practice, or to the fact that it was not powerful enough to provoke the adequate cortical change, detectable through a behavioural response or to transfer the learning to other skills (Lee, Seo, & Jung, 2012).

On the other hand, the reasons why the stimulation did not affect the sRT compared to Go-RT could be the lower complexity of the task which did not benefit the potential enhancement of the cortical excitability. In the same way, the stimulation parametres over the left-DLPFC might be not crucial for affecting sRT. Perhaps, by stimulating the premotor or motor areas, directly involved in sRT task, we would have obtained an improvement in this task too.

Additionally, we tracked all participants training daily-results showing they all improved; unfortunately these results did not have such accuracy to be considered reliable values for statistical analyses, which, on the other hand, may have been helpful for a deeper understanding of the online effect of tRNS combined with cognitive and motor training (Santarnecchi et al., 2015).

In contrast to other studies that found an improvement from the combination of tRNS and motor or cognitive training (Brem et al., 2018; Cappelletti et al., 2013; Prichard et al., 2014; Santarnecchi et al., 2015; Snowball et al., 2013) this study didn't show a better performance when the two techniques were coupled, at least in the two tasks investigated. Therefore, we can only deduce that this combined protocol does not affect sRT and Go/NoGo.

In this study we tested just sRT and Go/NoGo without finding a coupled effect; more tests should be added in order to investigate any potential cognitive improvement due to the training coupled with hf-tRNS, especially considering the significant improvements achieved by the effect of the exergame and the stimulation independently.

Summarising, we found a double dissociation where tRNS over the left-DLPFC can acutely improve performance in the Go/No Go task making healthy young adults faster preserving their accuracy, while the exergame training shows its efficacy in reducing sRT but not Go-RT.

This is the first exploratory study investigating the effects of 8-sessions of hf-tRNS and exergame training, alone and combined. Despite long-term effect and potential transfer to other cognitive tasks needs to be implemented, we suggest that exergame

training, which combines cognitive and physical demands in a motivating and enjoyable activity, might be an effective way to promote physical and cognitive exercise. Thanks to the low cost and the home-based nature, in a preventing perspective, it could be explored as part of a cognitive and motor initiative for active ageing!

We found promising results that encourage the use, even prolonged, of noninvasive electrical stimulation. Regarding its safety, it did not show any side effects (like cutaneous perception) which makes it the best research option for the Sham condition.

These encouraging results suggest the potential cognitive and motor benefits of using these tools even for several practical applications such as professional training, athletic training, and rehabilitation training.

## 4.5. Acknowledgements

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# CHAPTER 5. GENERAL CONCLUSIONS

Neuroplasticity is the mutual expression of the relation between the brain and the environment. The preciousness of our brain of being plastic by promoting functional organisation throughout the whole life offers a great and very flexible opportunity of intervention.

Basing on the ability of the brain to be shaped by experience, this thesis project aimed to design and develop different experimental procedures in order to promote and investigate short- and long-term plasticity effects using non-invasive brain stimulation techniques.

Few studies have explored the impact of the various stimulation parameters on cortical functioning, precisely the effect of the frequency band selected for the stimulation protocol. Despite Terney et al., (2008) reported a significant increase with high-frequency spectrum without any changes in corticospinal excitability by applying low frequencies (0.1 - 100 Hz), and Fertonani et al., (2011) observed a better behavioural performance in high-frequency band condition, there are no scientific reasons why we commonly use high frequency ranges from 100 to 640 Hz (this was in fact due to a limitation of the device used) and above all, it is still unknown why the whole high-frequency band induces an increase in cortical excitability.

Further investigations on hf-tRNS application and its mechanism of action on neuroplasticity are required. With this purpose, we created the first experimental design, targeting the motor cortex whose cortical excitability can be easily investigated by eliciting MEPs using TMS (Barker et al., 1985).

We aimed to explore possible differential modulatory effects of hf-tRNS on cortical excitability, creating different high-frequency bands, precisely splitting the total high-frequency spectrum creating two new sub-ranges: a lower band (100 - 400 Hz) and a higher band (400 - 700 Hz). Then, we compared the effect of these two sub-ranges of frequency to Sham stimulation, and in a second experiment, we delivered the whole high-frequency band (100 - 700 Hz) comparing the effects with those obtained with Sham stimulation.

Interestingly, the two narrow halves hf-tRNS caused a very tiny effect, without showing the ability to impact the cortical excitability of M1. We hypothesised that, by reducing the range of frequencies, we also reduced the amount of noise, which, following the stochastic resonance theory, might be the responsible of boosting the sensitivity of the neurons, as a result of a lack of increase of signal-to-noise ratio (Miniussi, Harris, & Ruzzoli, 2013; Terney, Chaieb, Moliadze, Antal, & Paulus, 2008). Consistent with this explanation, the results obtained in the second experiment showed an increase of cortical excitability up to 20 minutes after stimulation thus, tRNS was able to induce a lasting plastic effect. With respect to previous studies by Terney and Moliadze and colleagues who found a long-lasting effect up to 60 minutes (Terney et al., 2008; Moliadze et al., 2014) and Laczó and colleagues, who found up to 40 minutes (Laczó et al., 2014), in our study we obtained a shorter effect. We supposed that the differences in duration of cortical excitability enhancement found could have depended on the different parameters of the electrical stimulation such as current type, current intensity, duration of stimulation, stimulation site, frequency range.

The crucial indication we gained from this tRNS study is that a large amount of noise (i.e. a wide range of frequencies) is needed to produce a substantial and persistent increase in cortical excitability, while a smaller amount of noise (i.e. a narrower frequency range) does not produce such a modulatory effect.

In the second study, we investigated the effects of the stimulation when applied on low-level visual functions. We combined hf-tRNS, previously demonstrated being the optimal frequency range to enhance cortical excitability, or Sham stimulation, with the effect of 8 sessions of perceptual training, in improving visual acuity and contrast sensitivity in two groups of patients with anisometric amblyopia.

Basing on previous behavioural training interventions which proved positive results in both VA and CS functions (Polat et al., 2004; Zhou et al., 2006), the present study moved towards a combined approach in the rehabilitation of visual defects adding the brain stimulation. The goal was, by boosting neural visual plasticity, to enhance the effects of existing behavioural regimes (Spiegel et al., 2013b; Thompson et al., 2008), and to reduce the treatment protocol length since most of them are time-consuming (e.g. from 30 to 80 sessions; Polat et al., 2004), and require monitoring the treatment progress over long periods (at least  months) (Tsirlin, Colpa, Goltz, & Wong, 2015).

The short perceptual training resulted in an overall improvement of CS (the trained function) in both groups. However, for what concerns VA, an improvement was observed only in the group that received both the training and the real electrical stimulation with an improvement of about 0.2 LogMar. This finding gives evidence for a transfer to untrained visual functions such as VA induced by hf-tRNS when applied over early visual areas, with a long-lasting effect up to 6 months.

Another interesting finding of the present study was the transfer of improvement of VA (in the tRNS group only) and CS (in both groups) to the untrained healthy eye; this result is in line with other studies investigating the effects of PL with and without brain stimulation of the amblyopic visual cortex (Polat et al., 2004).

Amblyopia is associated with impairments in both monocular and binocular vision with an inefficient intraocular communication (McKee, Levi, & Movshon, 2003). Since binocular cells and their connections in the amblyopic visual cortex seem to be actively suppressed rather than absent (Hess et al., 2011; Mansouri, Thompson, & Hess, 2008), this result suggests that plastic changes also occurred at the binocular level.

We speculated that the encouraging results obtained in the present study might either be due to an increase in excitability of connections leading to the suppressed eye, likely by reducing the GABAergic inhibition from the unaffected eye, or by increasing response of glutamatergic connections (Jiao et al., 2011; Zheng & Knudsen, 1999).

Beyond the mechanism of action which requires further investigation, our results reinforce the concept that the mature visual cortex possesses a significant amount of plasticity and that visual functions can be improved even passed the critical period of visual development.

In line with the optimistic last finding, in the third study we maintained high-frequency band (100 - 640 Hz) as the preferential range to increase cortical excitability, but this time we investigated its action on high-level functions, precisely on inhibitory control in young adults. We chose the entertaining exergame “Dr. Kawashima, body and brain exercises” which, differently from the traditional videogame, promotes an active lifestyle since it is based on the combination of physical and cognitive activity.

It has been demonstrated that specific cognitive training is more effective if performed in combination with physical exercise (Eggenberger et al., 2015). The benefits have been shown not only at the behavioural level but also at functional and structural ones, reporting changes in cortical volume in the motor and frontal areas, supporting that physical activity enhances brain functioning (Kramer & Erickson, 2007; Kramer et al., 1999).

Knowing that brain plasticity is the basis of learning, and tES may be one way to contribute to increasing the learning rate, we investigated the effect of 8-sessions of hf-tRNS, 8-sessions of exergame and the two procedures combined. By testing these two protocols individually or coupled, we could isolate any potential effect of each one as well as any synergistic effect caused by their interaction.

As previously demonstrated, the combination of tRNS with cognitive training led to a significant improvement in the speed of learning (Brem et al., 2018; Cappelletti et al., 2013; Snowball et al., 2013).

We applied hf-tRNS on the left-DLPFC, shown being involved in inhibitory control (Wessel, 2018), and before and after the treatment, the participants performed sRT and Go/NoGo tasks.

Surprisingly, we found a double dissociation where the hf-tRNS improved RTs of Go trails, making the players faster while preserving their accuracy, whereas the exergame training showed its efficacy in reducing simple RTs.

This pilot study investigating 8-sessions of hf-tRNS and exergame training effects, individually or coupled, suggests that exergaming, which combines cognitive and physical demands in a motivating and enjoyable activity, might be an effective way to

promote physical and cognitive exercise. Regarding the functions assessed, it has been demonstrated useful in reducing reaction times. On the other hand, tRNS, once again, could have played a fundamental role by enhancing the excitability of DLPFC and improving the cognitive processes involved in this task (Takeuchi & Kawashima, 2012).

No significant effects have been shown combining the two techniques, likely due to the already high-performance level obtained in the tasks, which decreases the possibility to obtain any additional improvements.

The literature on NIBS methods is growing exponentially: the increasing popularity goes with their enlarged use in association with behavioural training. Critical debates about their trustworthiness and potential reliability for clinical or applied use are overwhelmingly rising and there is still a lack of guidelines for evaluating and interpreting methodological aspects of transcranial stimulation. Also, tES are relatively new techniques when applied to investigate the relation between the brain and the behaviour, indeed more evidence from neuroimaging to understand brain activity modulation is needed.

Although recently tRNS has been used and compared to the other tES protocols for cortical excitability modulations in humans, knowledge regarding safety parameters such as the current intensity, the size of the electrodes and the duration, is so far limited.

The most frequent side effect, especially for tDCS, is the tingling sensation (70.6%) (Poreisz et al., 2007), and even though it has been shown that tRNS has overcome this

limitation (Ambrus et al., 2010), we still do not know if it induces long-term side effects, due to the absence of longitudinal studies.

In two of the studies described in this manuscript, an excellent tolerance of 8-session tRNS of 25 minutes each has been ascertained: no participant reported side effects, and none was able to feel any cutaneous perception of the stimulation. This is an important contribution to the requirement of safety guidelines, considering that the procedures created consisted of several sessions of consecutive daily stimulation in combination with behavioural training.

Many approaches have been tested to create effective non-invasive treatments and, even if tRNS mechanism of actions are still unclear, this promising technique represents a useful new tool in understanding how neural plasticity can be exploited to obtain behavioural improvements.

To conclude, this manuscript contributes to the understanding of the application of electrical stimulation on the cerebral cortex to enhance cortical excitability and to boost the effect of behavioural training. TRNS, as expression of neural plasticity, showed the possibility of inducing beneficial effects, and it provided reasonable expectations about the long-term aim of developing new strategies to promote cognitive enhancement, recovery and likely, to boost the effects of therapeutic intervention in several clinical populations.

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