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The synergistic effect of combining physical activity and anodal tDCS boosts motor cortical interhemispheric plasticity in mice

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ABSTRACT

In recent years, transcranial direct current stimulation (tDCS), one of the most encouraging non-invasive neuromodulatory techniques, has been combined with other types of interventions, such as physical or cognitive training. The idea is that the coupling of tDCS and an endogenous brain activation (e.g. motor activity) may have a synergistic addictive effect with respect to single interventions. Nevertheless, the mechanisms responsible for this synergistic effect are not clear and animal studies on this topic are still missing.

In both humans and rodents, anodal tDCS (A-tDCS) increases neural activation and plasticity of the primary motor cortex (M1). However, the effects of combining M1 A-tDCS and physical activity have not been studied yet. Moreover, the motor cortices are highly interconnected, and this thesis is based on the hypothesis that tDCS might affect motor cortex functional connectivity when coupled with its physiological activation. For these reasons, we evaluated the effects of the combination between monolateral A-tDCS and moderate physical activity in the stimulated and non-stimulated motor cortices, in C57/Bl6 young mice. To this aim we measured cell activation by the immediate early gene (IEG) c-Fos expression, functional connectivity by local field potential (LFP) M1-M1 coherence analysis and structural plasticity by the mean spine density with Golgi-Cox technique.

We found that, when A-tDCS is applied in combination with walking, mice displayed higher cell activation and mean spine density in layer II/III, in the directly stimulated hemisphere but also in the contralateral M1. Functional connectivity is also affected by the coupling, with an enhanced M1-M1 synchronization in the theta rhythm, which is associated to locomotion. This inter-hemispheric boosting effect does not occur when mice receive the stimulation alone, supporting the idea that tDCS effects strongly depend on the ongoing network activity. Thus, motor activity physiologically activates the motor network which, in turn, determine the tDCS-induced plasticity effects. All this data sustains the idea that coupling tDCS and endogenous activation leads to the synergistic boosting effects of the coupling, as observed in human studies.

Of note, physiological aging has been associated to a reduction of M1 plasticity and changes in tDCS-induced plasticity effects in humans. Hence, the second aim of this investigation was to verify the eventual synergistic effects on cell activation and structural plasticity in elderly mice. Our findings reveal that both the activation and the mean spine density in layer II/III resemble the ones in young adult mice, indicating that the synergistic effects of the combo are still efficacious, even if the motor network plasticity is physiologically reduced.

In conclusion, the application of monolateral M1 A-tDCS during physical activity results in a widespread motor cortex increased plasticity.

1. INTRODUCTION

1.1 Non-invasive neuromodulatory techniques

In the last decades, non-invasive neuromodulatory techniques have been receiving growing attention as tools to effectively treat neurological and psychiatric disorders, with the great appeal of bypassing the adverse effects associated with invasive approaches (Camacho-Conde et al., 2022; Fregni & Pascual-Leone, 2007; Johnson et al., 2013; Tortella, 2015).
The term '*neuromodulation*' means the interaction with the brain, via electrical, magnetic, chemical, or optogenetic methods, with the goal of long-term modifications or regulation of the neural activity (Krames et al., 2009). Currently, non-invasive neuromodulation techniques have been proven to ameliorate symptoms in depressive disorders, chronic pain, aphasia, movement disorders (as Parkinson's disease), motor stroke, epilepsy, disorders of consciousness, Alzheimer's disease, tinnitus, schizophrenia, substance abuse, addiction and craving, amongst others (Johnson et al., 2013; To et al., 2018).

The two most commonly adopted non-invasive neuromodulatory techniques are transcranial magnetic stimulation (TMS) and transcranial direct current stimulation (tDCS) (***Figure 1***). TMS is based on the induction of single or repetitive (rTMS) pulses of electric current within the brain, obtained by generating magnetic fields near the scalp through the application of an electromagnetic coil. tDCS involves the application of a weak continuous electrical current flowing between two electrodes, the cathode and the anode (Rothwell, 2018).
With respect to conventional brain stimulation methods, such as deep-brain stimulation, epidural stimulation or electroconvulsive therapy, these non-invasive brain stimulation (NIBS) techniques are safer and do not require hospitalization. In addition, tDCS presents many favorable advantages over rTMS in terms of portability, reduced costs, and ease of combination with other treatments (e.g., rehabilitation sessions) (Peruzzotti-Jametti et al., 2013).

Considering the density of the current applied, tDCS can produce only small electrical currents in the brain which are not sufficient to generate action potentials in neurons, contrarily to TMS technique. Thus, tDCS is considered a pure neuromodulatory technique, whereas TMS can be both neuromodulatory and neurostimulatory (if supra-threshold stimuli are given) (Rothwell, 2018; Williams et al., 2009).

Figure 1. tDCS and TMS apparatus. On the left, the tDCS apparatus. It comprehends a device for the current supply and two electrodes put on the scalp. On the right, the TMS equipment. It is composed by a capacitor for the generation of a powerful and rapid-discharging electrical current, which flows into a figure-of-eight coil placed on the scalp. Images taken by Wikipedia.

Other NIBS techniques that have been developed in the last few years are the transcranial alternating current stimulation (tACS), which employs oscillatory electrical stimulation to facilitate neuronal activity in specific frequency bands, the transcranial random noise stimulation (tRNS), which focuses on the existing link between behavior and frequency-specific noise inherent in neural processing, and the transcranial focal ultrasound stimulation (tFUS) that transmits low-intensity ultrasound on deep brain regions (Polanía et al., 2018; Zhang et al., 2021).

In terms of spatial resolution, TMS and tDCS affect a relatively large neuronal population although it is generally accepted that their spatial resolution may be task-related. This refers to the hypothesis that the stimulation may have different effects on neurons that are at rest or activated by an ongoing behavior (Kar & Krekelberg, 2012; Polanía et al., 2018).

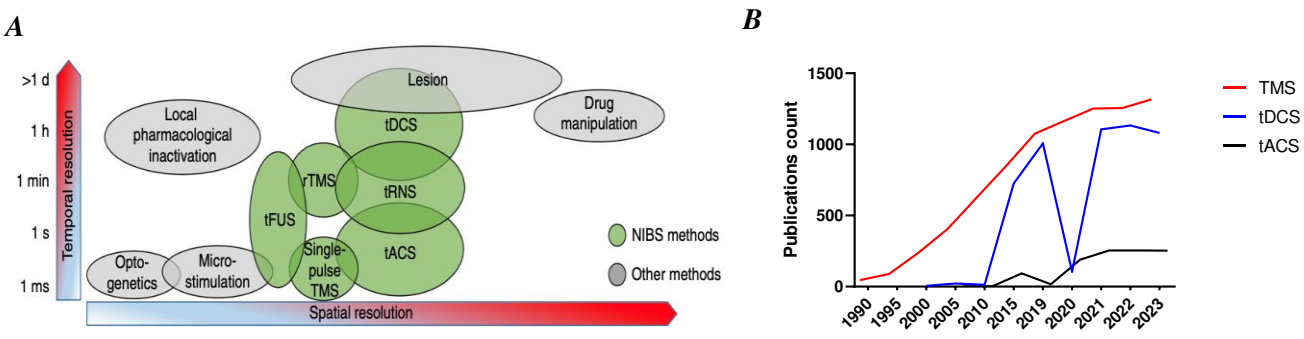

Figure 2. Non-invasive brain stimulation methods. A. The temporal and spatial resolutions of the different techniques. In green: the NIBS techniques; in grey: other methods. Image adapted from Polanía et al., 2018. ***B.*** The growing number of citations per year of NIBS techniques, from 1990 to 2023. The number of publications per year were taken by searching for 'TMS', 'tDCS' and 'tACS' on Pubmed website.

The temporal resolution of the single-pulse TMS is optimal, namely milliseconds (Ward, 2015) (***Figure 2A***). On the contrary, the temporal resolution of tDCS is low, as the neuromodulatory effects start occurring after few seconds from the beginning of the stimulation, however the effects continue throughout the interventions and tDCS physiological after-effects can last for hours or even days instead. Other transcranial electrical stimulation techniques, as the tACS and tRNS, have a better temporal resolution (Polanía et al., 2018). Lastly, tFUS has the better spatial and temporal resolution in respect to the other available NIBS (Zhang et al., 2021).

Despite their versatility and popularity (***Figure 2B***), the mechanisms underlying the efficacy of NIBS techniques remain quite uncleared (Cirillo et al., 2017; Polanía et al., 2018). Of note, though the concept of modulating brain activity with electrical current has been used for centuries, its effects on the brain are not totally unveiled yet.

1.2 Transcranial direct current stimulation (tDCS)

1.2.1 A brief history of tDCS

The electrical stimulation had been used to treat diseases for centuries.
The first source of electricity was the animals' electricity and indeed Antique Egyptians knew about the electrical properties of the Nile catfish, though it is not clear if they use the animal for clinical purposes. The first actual evidence of electrical stimulation dates to the Greece times, when Plato and Aristotle reported the ability of the torpedo fish to generate curative effects by its electrical discharge (Althaus, 1860; "Book Review Electrical Treatment. By Wilfred Harris, M.D., F.R.C.P. 12mo, Pp. Xii, 384. With 25 Illustrations. Chicago: W. T. Keener & Co. 1908.," 1908; "Recent Literature The Medical and Surgical Uses of Electricity. By A. D. Rockwell, A.M., M.D. Illustrated with 200 Engravings and One Plate; 8vo; Pp. Xvi, 612. New York: Wm. Wood & Co. 1896.," 1897). It is only in the Roman Empire times, however, that the physician Scribonius Largus placed a live torpedo fish over the scalp to relieve a patient's headache, giving birth de facto to the transcranial electrical stimulation (Cambiaghi & Sconocchia, 2018). Later, in the 11th century the Muslim physician Ibn-Sidah proposed to treat epilepsy by placing the torpedo fish over the brows of the patients (Priori, 2003). The electrical stimulation also spread in Africa, with the Jesuit missionaries using the catfishes to expel 'devils out of the human body' (Parrish, 2007). Despite the early, world-spread, use of

torpedo fish, it was necessary to wait until the 1773 to discover its properties, with the in-depth study by John Hunter that demonstrated that the so called 'shocks' induced by the animal were caused by the generation of electricity (Walsh & Seignette, 1771).
The historical traces of tDCS drive to many centuries later, when Otto von Guericke invented the first electrostatic generator, considered the first stimulator device, in 1660 (Comroe & Dripps, 1976). Years later, its variation was used by the Italian scientist Marco Antonio Caldani to stimulate muscles in sheets and frogs in 1756 (Sarmiento et al., 2016). Contemporarily, the first capacitor called the Leyden jar was developed in 1745 by Georg von Kleist. Thus, Anton de Haen and Benjamin Franklin were able to combine electrostatic generators and the Leyden jar for therapeutic electrification (McWhirter et al., 2015).

Although they were the first kinds of electrical stimulation in history, neither the fish electricity nor the electrostatic electricity implies a direct current (DC), meaning the flow of an electrical charge that do not vary in time (Sarmiento et al., 2016). It was only in the 18th century, in fact, that Luigi Galvani invented a DC battery, called galvanic battery, that was later used by his nephew, Giovanni Aldini, for clinical applications (***Figure 3***). Aldini utilized DC to treat the melancholy madness (major depression) of the 27-year-old farmer Luigi Lanzarini. He observed that the mood progressively improved and several weeks after the beginning of the treatment the patient apparently resulted cured (Parent, 2004). The work of Aldini was the pillar from which the direct current stimulation (DCS) era begun.

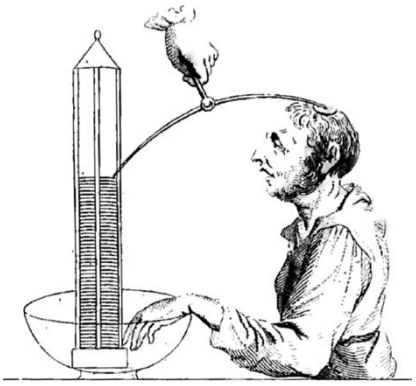

Figure 3. Aldini's patient receiving the treatment with the electrical circuit completed by the patient's hand touching the base of the Voltaic pile. Image adapted by Aldini, 1803.

Around 1880, DCS was particularly popular among German psychiatrists as many researchers used the DCS for the treatment of mental disorders during the 19th and the 20th century (H. Steinberg, 2013). However, the variable and inconclusive outputs led to the abandon of this methodology.

Historically, the attempts to stimulate the brain with current through the scalp did fail (Merton & Morton, 1980), whit just a successful report by Gualtierotti and Peterson in 1954. Actually, these researchers managed to obtain contralateral motor responses when applying trains of stimuli over the motor cortex, as those in the exposed cortex, of both anesthetized baboons and men (Gualtierotti & Paterson, 1954). The reason of these failed attempts was due to the attachment of both the electrodes on the scalp surface resulting not only in a painful sensation, as most of the current flowed through the skin between the electrodes, but also in a quite inefficient neurons activation, as a smaller current fraction penetrated the skull and scalp (Rothwell, 2018).

In 1930s, Cerletti popularized the electroconvulsive therapy (ECT) with the successful treatment of a patient with schizophrenia. It consists in a strong current pulse which causes a seizure in Cerletti's patient (Lewis et al., 2016).

Lately, DCS reappeared in 1964 at first with animal studies and successively with the study of Lippold and Redfearn, reporting that anodal current improved the alertness, mood and motor activity in healthy subjects whereas cathodal current induced quietness and apathy (Lippold & Redfearn, 1964). From the 1970s DCS was abandoned again because of the introduction of new psychiatric drugs (Dubljević et al., 2014).
Finally, in 1980 Merton and Morton developed a stimulator that worked at high voltage with the aim of reducing the skin resistance to allow the current to better penetrate the brain and they successfully induce contralateral body muscles contraction by stimulating the motor cortex once (Merton & Morton, 1980).
It is only in recent years, however, that the works of Priori in Milan and Nitsche & Paulus in Gottingen have made it clear that DCS can be used as a non-invasive technique to effectively modulate the human brain (Nitsche & Paulus, 2000; Priori et al., 1998).

1.2.2 tDCS: how it works, properties & characteristics

tDCS popularity has grown considerably over the past decades, as exemplified by searching the word 'tDCS' in Pubmed site: from 65 articles published between 2000 and 2005, to 1500 articles published between 2011 and 2016, until reaching 4800 articles published in 2019-2023 years (see also **Figure 2B**). This increased attention depends on its ability to modulate not only neural activity but also entire

brain networks and functional connectivity in a non-invasive manner (Lewis et al., 2016; To et al., 2018). Additionally, tDCS long-lasting (or 'offline') effects, outlasting the stimulation time, are also receiving a huge interest, especially for clinical purposes (Nitsche & Paulus, 2000).

The applications of tDCS technique attain many fields, from research purposes to the neuro-enhancement and to symptoms relief in neurological and other pathological and psychiatric conditions.

tDCS has already been applied, in fact, in several clinical trials to alleviate symptoms in neurological disorders, as Parkinson's and Alzheimer's diseases, prompting its efficacy in improving cognitive and motor impairments. Currently, its beneficial clinical effects are being also investigated in trials with patients suffering forms of epilepsy, autism, Down syndrome and stroke (Camacho-Conde et al., 2022). Moreover, tDCS has been applied to psychiatric illness such as major depressive and bipolar disorders, schizophrenia and others, with promising even though not resolutive results. In literature, a few studies also concern the application of tDCS for the treatment of substance dependencies by stimulating the prefrontal cortex (Tortella, 2015).

In recent years, a growing body of studies centered their attention on tDCS as a promising technique to enhance athletic performance. The positive trials have prompted Davis to coin the word 'neuro-doping' which indicates the use of advanced techniques for mental and physical enhancement of athletes in 2013 (Davis, 2013).

For research purposes, tDCS has been deployed in the study of the functional roles of specific brain regions and the exploration of large-scale neural network dynamics (Cirillo et al., 2017; Polanía et al., 2018).

During the stimulation, the current flows between the electrodes, from the positive pole (anode) to the negative pole (cathode), passing through the brain to complete the circuit. In the unipolar setting, one electrode is known as the 'active' electrode, and it is placed on the scalp whereas the other 'reference' electrode is located extra-encephalically.

tDCS 'polarises' neurons by the modulation of their resting membrane potential: anodal stimulation depolarizes the neurons, increasing the probability for an action potential occurring; cathodal stimulation hyper-polarizes neurons, decreasing the chance for an action potential (Nitsche et al., 2008; Nitsche & Paulus, 2000; Thair et al., 2017).

It is commonly accepted that an anodal current can temporarily facilitate behavior of the cortical region under the electrode, whereas a negative cathodal current inhibits behaviors (Nitsche et al.,

2008), and, although the polarizing effects are restricted to the area under the electrode, functional effects can be observed even in other interconnected brain areas (Vines et al., 2008).

According to computational studies, a single neuron receiving extracellular DCS will have some depolarized compartments and others that become hyper-polarized (Bikson et al., 2004; Chan et al., 1988; Jackson et al., 2016). In ***Figure 4A***, it is represented a cortical pyramidal neuron having the apical dendrites nearer the anode and its basal dendrites farer from it. When the current is flowing, there is an inward current generation that causes the neuron polarization. This latter differs across the neuronal segments according to their location in respect to the anode. In this case, the apical dendrites near the anode hyper-polarizes and contemporarily the soma and basal dendrites of the neuron depolarize.

Thus, the neuron orientation in respect to the electric field and its morphology are fundamental for tDCS effects on neuronal polarization, as indicated in ***Figure 4B***.

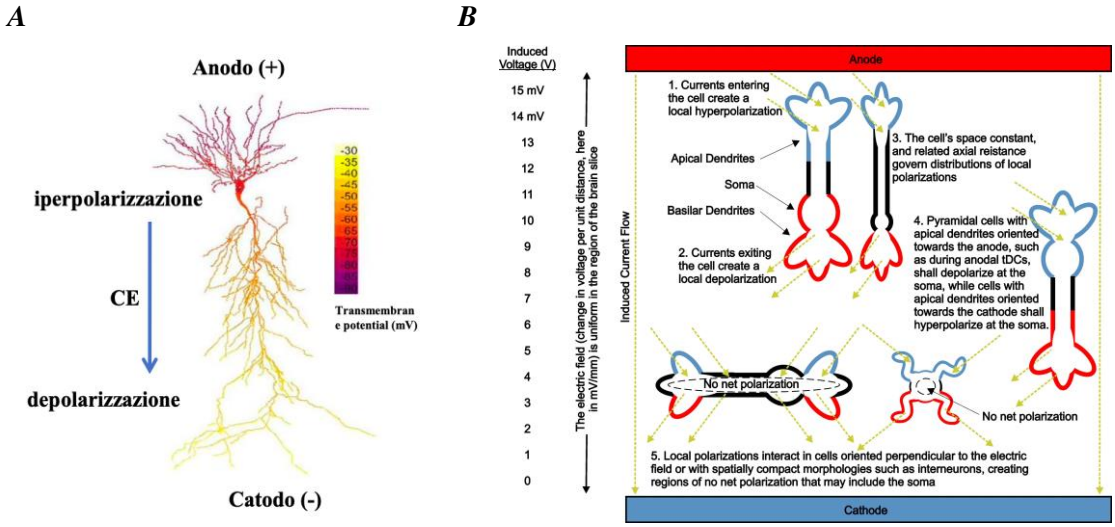

Figure 4. A. Schematic representation of the polarization occurring in a neuron placed within a current flowing from the positive to the negative pole. ***B***. Stylized images of cortical pyramidal neurons differently oriented to the anode. Images adapted by Radman et al., 2009.

The efficacy of tDCS depends on the current flow direction (anodal or cathodal stimulation) and on the current density, which determines the induced electrical field strength. The current density results from the quotient of current strength and electrodes size. It is commonly agreed that beneath a certain current density (0,017 mA/cm^2), the stimulation is not effective (Nitsche et al., 2008).

Considering the electrode sizes, Nitsche et al. showed that tDCS effects can be focalized by reducing the size of the stimulating electrode. On the other hand, by increasing the size of the reference electrode, it is possible to render it functionally ineffective.
Other important parameters are the duration of the stimulation (a longer stimulation will induce after-effects outlasting the end of the stimulation), and the orientation of the electric field (for the reasons discussed above) (Nitsche et al., 2008).
The gender is also an important factor, for example, studies revealed that cathodal tDCS is more effective in woman and that anodal tDCS (A-tDCS) in the visual cortex affected more women than man (Chaieb et al., 2008; Kuo et al., 2016).

1.2.3 Effects of tDCS in physiological conditions

Despite its long history in medicine, the mechanisms of action and the effects of tDCS are still incompletely unveiled (Jackson et al., 2016).
To understand its underlying action, electrophysiological and pharmacological approaches have been widely applied to the motor and to the visual systems, both in humans (Antal et al., 2001, 2003; Liebetanz et al., 2002; Nitsche et al., 2008; Nitsche & Paulus, 2000; Notturno et al., 2014; Ranieri et al., 2012; F. Steinberg et al., 2019) and animals (Barbati et al., 2020; Bindman et al., 1964; Cambiaghi et al., 2010; Creutzfeldt et al., 1962; Fritsch et al., 2010; Gellner et al., 2020).

In animal studies in the 1960s, it had been observed that tDCS was able to modify cortical excitability. In those investigations, it was showed that DCS on the cortical surface specifically modulates the resting membrane potentials in a polarity specific manner, and, in turn, this causes alterations in the spontaneous neuronal discharge rates (Bindman et al., 1962, 1964; Gorman, 1966; Purpura & McMurty, 1965).
Successively, it was observed in humans that tDCS induces excitability changes in the motor cortex through the analysis of motor-evoked potentials (MEPs) (Nitsche & Paulus, 2000, 2001). Particularly, it was noticed that the application of A-tDCS over the motor cortex increases the excitability of the underlying area, whereas cathodal stimulation decreases it (Nitsche & Paulus, 2000). Moreover, the effects extend beyond 1 hour when tDCS was applied for more than 10 minutes, outlasting the stimulation time (Liebetanz et al., 2002; Nitsche & Paulus, 2001).
More recently, the same results were resembled in animal studies. MEPs evoked by tDCS were recorded from the forelimb of mice under anesthesia, before and after 10 minutes of anodal and

cathodal tDCS. This study demonstrated that MEPs sizes are significantly increased promptly after A-tDCS and reduced after cathodal tDCS, in respect to sham conditions. Both effects decline towards basal levels in the following 10 minutes (Cambiaghi et al., 2010).

tDCS long-lasting changes may be significantly relevant in cognitive/therapeutical applications (Yoon et al., 2012), however these after-effects have not been completely explained.

The long-lasting effects of tDCS on neural excitability may suggest the engagement of plasticity-like mechanisms (Cirillo et al., 2017; Nitsche et al., 2003) and early human pharmacological studies have suggested that they could be dependent on NMDA receptors (D'Angelo & Rossi, 1998). Liebtanz et colleagues tested the effects of some molecules able to block membrane ion-channels and NMDA receptors in healthy subjects. Their results brought them to propose that, in case of A-tDCS, the membrane depolarization accompanied by an increased firing rate could lead to an NMDA receptor-mediated augmentation of synaptic strength, presumably via higher intracellular calcium levels (Liebetanz et al., 2002). One year later, other pharmacological studies performed by Nitsche et al. supported the previous hypothesis, suggesting that the short-lasting effects may be due to membrane polarization, whereas the long-lasting after-effects could depend on NMDA receptors activity (Nitsche et al., 2003).

Since these first observations, an *in vitro* study of Fritsch et colleagues demonstrated that anodal direct current stimulation (DCS) applied to M1 rodent slices effectively induces NMDA receptor-dependent long-term potentiation (LTP), a form of activity-dependent long-term changes in synaptic efficacy (Malenka & Bear, 2004). Notably, this result was obtained only when DCS is combined with a concomitant synaptic activation (Fritsch et al., 2010).

Then, several animal studies have provided evidence that A-tDCS enhances LTP at CA3-CA1 synapses (Podda et al., 2016; Ranieri et al., 2012; Rohan et al., 2015).

Beyond these functional plasticity changes, it has emerged from recent evidence that the structural plasticity is also affected by tDCS method. The term structural plasticity refers to the modifications in the number, location, and size of spines, dendritic and axonal branching patterns (Bozelos & Poirazi, 2017).

In a 2020 study, mice treated with 3 daily sessions of A-tDCS over the motor cortex not only show an increase LTP along with an enhanced basal synaptic transmission in layer II/III horizontal connections, but also a higher dendritic spine density in layer II/III pyramidal neurons of the primary motor cortex (Barbati et al., 2020). In another animal study, the A-tDCS applied over the primary motor cortex in combination with the contralateral electrical forepaw stimulation rapidly increases the spine density and the changes last for 24 hours after the stimulation. Specifically, this intervention

on anesthetized mice induces both an increase of the original spine survival and the formation of new spines, morphologically characterized by larger head sizes (Gellner et al., 2020).
In a rat model of cerebral infarction, it has been demonstrated that A-tDCS induces an increased spine density after stroke, promoting structural plasticity and the recovery after stroke (Cirillo et al., 2017; Jiang et al., 2012).

1.2.4 Brain state dependency of tDCS effects

Although tDCS is a neuromodulatorry technique, evidence of tDCS implications in firing rates and in synaptic efficacy were observed in both animals (Bindman et al., 1962) and humans (Nitsche & Paulus, 2000; Priori, 2003).
The emerging idea is that tDCS-induced effects may depend on the ongoing activity of the system when the stimulation is applied.
For the last 30 years, in fact, tDCS has been applied without considering the internal state of the brain when the stimulation was supplied. However, neurons in a brain region are not isolated but integrated into an active neuronal network (i.e. oscillations) and neurons are strongly influenced by the temporospatial dynamics of spontaneous network activity, which is settled by rhythmic fluctuations in neural excitability under the control of ascending pathways and cortico-cortical projections (Draguhn & Buzsáki, 2004; Schroeder & Lakatos, 2009). Evidence suggests that the frequency, amplitude, and phase of the neural oscillations lead to transient, local or even global network states that determine not only the response to incoming sensory stimuli (Sadaghiani et al., 2010; VanRullen & Koch, 2003) but also the immediate as well as the long-lasting effects of tDCS. This leads Bergman to forge the term ‘brain-state dependent brain stimulation’ (Bergmann, 2018).
This neural co-activation (tDCS + active brain network) has been also proposed to be the key responsible for the synaptic plasticity induction (Cirillo et al., 2017; Jackson et al., 2016; Rothwell, 2018).

Hence, if it is assumed that a neuron is near the threshold for the action potential generation as consequence of its network activity, then a relatively small depolarization (as the one introduced by tDCS) could be sufficient to trigger an action potential. In turn, these activated neurons may trigger action potentials in other neurons of the interconnected network, inducing changes also in the functional connectivity via tDCS application (Reato et al., 2010).

According to Bikson et colleagues, the brain state dependency of the tDCS could blur the distinction between 'supra-threshold' (as TMS) and 'sub-threshold' (as tDCS) stimulations (Bikson et al., 2012; Jackson et al., 2016).

Currently, the behavioral outcomes of neurological and psychiatric diseases seem to be the results of alterations of the brain network and its connectivity and not deriving from an impairment of an isolated region. As consequence, neuromodulation research has also shifted to target the entire brain networks and not only a specific region (To et al., 2018).

The importance of the brain-state during stimulation triggered researchers to evaluate tDCS outcomes when combined with simultaneous endogenous brain activation in both rodents (Fritsch et al., 2010; Gellner et al., 2020) and humans (Wang et al., 2021). These studies have mainly targeted the motor cortex, as it is one of the most studied pathways and its output is easy to detect through MEPs or motor performances (Barbati et al., 2020; Bindman et al., 1964; Cambiaghi et al., 2010; Creutzfeldt et al., 1962; Di Lazzaro et al., 2012; Fritsch et al., 2010; Gellner et al., 2020; Liebetanz et al., 2002; Nitsche et al., 2003, 2008; Nitsche & Paulus, 2000, 2001; Notturno et al., 2014; F. Steinberg et al., 2019).

1.3 Mouse primary motor cortex

1.3.1 Overview of the mouse primary motor cortex (M1)

The primary motor cortex (M1) is the central hub responsible for overall motor decisions and movements (Yu & Zuo, 2011).
The organization of the mouse motor cortex was firstly mapped in a dystrophic mouse strain by Li and Waters with the intracortical microstimulation study of 1990. They described the motor cortex as extending approximately 4.5 mm in the anterior-posterior direction and 2.5 mm in the medio-lateral direction, with the representations of vibrissae, forelimb, hindlimb and face/tongue occupying the largest space. Their experiments indicated that mouse motor cortex is topographically organized but an individual body part having more than just one representation. For example, the forelimb and hindlimb have a dual representation (***Figure 5)***. However, it has emerged that the complexity is even more, as the same movement is '*multiply represented within the map in a mosaic fashion …',*

similarly to contemporary reports on cat and monkeys cortices. The researchers also noted an overlap of forelimb and hindlimb representation generally occurring at the border between the two areas, even not exclusively (li & Waters, 1991). Decades later, Tennant et colleagues resembled the results obtained by Li and Waters in C57BL/6 mice.

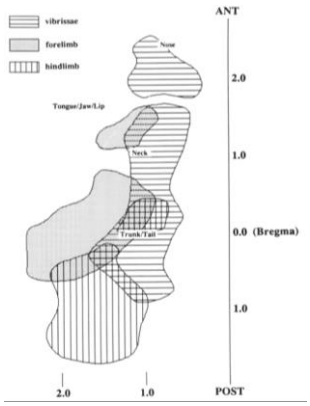

Figure 5. The idealized drawing of the location of motor representations (adapted from Li & Waters, 1991).

By considering the cytoarchitectonic aspect, mice have a 6-layered neocortex, which laminas are named from I to VI, starting from the surface of the cortex and going ventrally. Layers II/III are referred to as upper layers, vice versa layer V and VI constitute the deep layers (Suzuki & Hirata, 2013). Both the previously cited studies found that most of the motor map is comprehended in the so-called agranular lateral field (AGl) (***Figure 6***). The classification into granular and agranular is based mainly on the presence or absence of granular cells in layer IV, the extension of layer V and the degree of stained cells in layer II. AGl is characterized by a high concentration of cells in layer II, a wider layer V composed by big pyramidal cells and a layer IV containing just few cells. These findings are in line the previous study of Caviness about the cytoarchitecture of the mouse motor cortex (Caviness, 1975; li & Waters, 1991; Tennant et al., 2011).

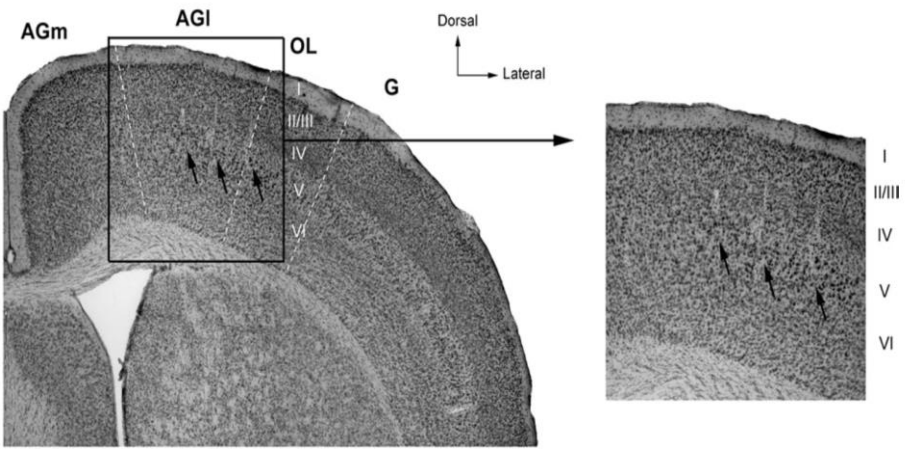

Figure 6. Nissl staining coronal sections with white dashed lines indicating different cytoarchitectural zones and Roman numerals referring to the layers. The black arrows point the end of the electrode for the intracranial microstimulation. AGm = medial granular cortex, AGl = lateral granular cortex, OL = overlapping zone; G = granular cortex. (Image adapted from Tennant et al., 2011).

1.3.2 Connectivity of the mouse M1

M1 is known to regulate movements and control motor learning through the enriched functional connectivity of the two motor cortices with various brain regions and with each other.

In the mouse neocortex, projection neurons are multipolar glutamatergic neurons, characterized by a recognizable pyramidal morphology (Molyneaux et al., 2007). These pyramidal neurons constitute the most abundant cellular subtype in the cortex (Tjia et al., 2017) and combine to form the intricate communication system of the motor cortex.
Pyramidal neurons in the upper layers, i.e. layer II/III, project to each other inside the cortex. Vice versa, neurons in deep layers project outside the neocortex to reach subcortical targets (***Figure 7***) (Suzuki & Hirata, 2013). In 1990, Li & Waters already pointed out, in fact, that all the cortico-spinal tract projections derived entirely from layer V of the M1 via retrograde tracing with horseradish peroxidase (HRP) (Li & Waters, 1991). Layer IV receives input from the thalamus and, subsequently, sends the information to the upper layers. This layered organization favors the local vertical flow of information among the densely interconnected layers and forms the vertical columnar unit (Suzuki & Hirata, 2013).

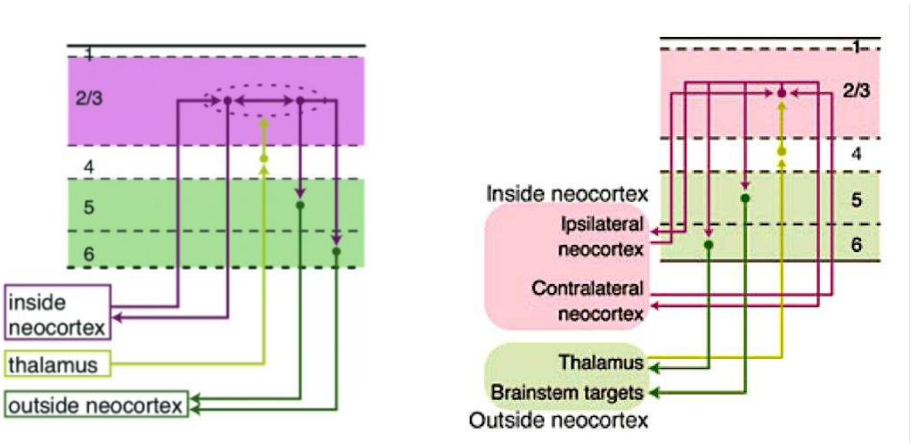

Figure 7. Schematic columnar neural circuit in the neocortex of the mouse. In magenta: the upper layers, in green: the deep layers (images adapted from Suzuki & Hirata, 2013).

The myelinated axons of the pyramidal neurons also form the corpus callosum and allow the integration of information between the two cortical hemispheres. Several reports mentioned that these transcallosal neurons mainly reside in layer II/III of the M1. Contrarily, other studies revealed that a significant amount of these contralateral projection neurons are placed in layer V.

A recent experiment with retrograde labeling supports the hypothesis that the transcallosal neurons are located in both layer II/III and laver V of the M1. In addition, the transcallosal projections show a layer-specific, columnar organization and most of them show a precise homotopic organization (Chovsepian et al., 2017). Thus, the contralateral connections pattern resembles the ipsilateral connections scheme, however, it has been proposed to be an attenuated but mirrored pattern (Goulas et al., 2017). Some transcallosal neurons of the deep layers can have dual projections, constituting a small proportion of heterotypic connections (Chovsepian et al., 2017).

1.3.3 Distinct properties of Layer II/III & layer V pyramidal neurons

Motor pyramidal neurons belonging to different cortical layers vary significantly in their dendritic morphology, functional properties and connectivity (Harris & Shepherd, 2015).

Morphologically, pyramidal neurons in layer II/III have smaller soma size and confined dendritic tree compared to layer V. The apical dendrites of layer V neurons extend a huge distance: they reach the surface of the cortex, sampling a greater area than neurons in layer II/III (Tjia et al., 2017).

Functionally, layer II/III neurons have lower spontaneous and evoked potential firing rate than those in layer V (Petersen & Crochet, 2013).

Finally, their axons project to distinct targets, as discussed in the previous chapter. Specifically, axons of pyramidal neurons in layer II/III terminate in neighboring and distant cortical areas, probably integrating information across cortical regions and mediating higher order information. Vice versa, neurons in layer V project to subcortical structures, as thalamus, striatum, midbrain and pons, along with the spinal cord and constitute the cortical output of the motor system (Harris & Shepherd, 2015; Tjia et al., 2017).

Thus, pyramidal neurons of layer II/III and layer V differ as they received distinct inputs. Beyond that, apical and basal dendrites of the same neuron might form synapses with different neuronal populations, varying the synaptic and spines dynamics (Feldmeyer, 2012; Tjia et al., 2017).

The postsynaptic sites of most of the excitatory synapses reside on dendritic spines, protruding from dendrites (Gray, 1959), indeed. In a recent study of 2017, it has been observed that density and the spines dynamic is higher in the apical dendrites of layer II/III than in layer V in young adult mice

(Tjia et al., 2017). No mention has been made about the basal dendrites in this paper, as in most literature studies concerning motor experience and dendritic spines.
Even the age should be considered as an important factor affecting dendritic spine dynamics: during adolescence apical dendritic spine density of layer V pyramidal neurons decreases due to an increase elimination to the detriment of spine formation. No net changes have been observed in neurons of layer II/III (Tjia et al., 2017).
Hence, from literature studies it seems that the layers may exhibit different motor experience-dependent dendritic spine remodeling and that spine dynamics of layer II/III pyramidal neurons is less known than the one in layer V (Ma et al., 2016).

1.3.4 Motor activity and M1 plasticity

Several literature studies indicate that motor training affects spines dynamics.
Even though dendritic spines in the adults' cortex are relatively stable, mice retain the possibility to learn new tasks during their lifespan and this presumably reflects modifications in the strength and number of synaptic connections (Tennant et al., 2012).
Considering the structural plasticity, previous studies have examined the spine dynamics in the motor cortex *in vivo* and the effects of motor learning on it. Xu et colleagues observed that training mice on a skilled reaching task promotes an immediate formation followed by a selective elimination of dendritic spines on the apical branches of layer V pyramidal neurons in the contralateral motor cortex in respect to the trained forelimb (Xu et al., 2009). Similar results in the spine dynamics of layer V apical dendrites were found in mice trained on accelerating rotarod (Yang et al., 2009). Both these studies also indicate that a continuous training is associated with an increase in the proportion of new spines that persist, contrarily to new pines in untrained controls (Xu et al., 2009; Yang et al., 2009). Later, Clark et colleagues resembled these previous findings and, by examining the maintenance of newly formed spines in mice with continuous training in respect to mice with only a brief training, they found that the sustained training is required for the preferential stabilization of spines that are formed in response to the motor activity (Clark et al., 2018). These results have raised the possibility that this preferential stabilization of newly formed spines during ongoing training is a mechanism for the long-term retention of motor skills (Clark et al., 2018; Xu et al., 2009; Yang et al., 2009). All the three evidence found no changes in the spine density of superficial apical dendrites of layer V neurons in both trained and untrained mice. In contrast, an increase in the spine density of apical dendrites in layer II/III pyramidal neurons was observed in the M1 of trained mice (Clark et al., 2018). However,

another recent evidence did find any difference in spine dynamics of layer II/III apical dendrites in mice during the motor learning of the single pellet reaching task, contrarily to layer V enhanced spine turnover (Tjia et al., 2017).

Nevertheless, the involvement of layer II/III neurons in motor learning was demonstrated through calcium imaging and LTP studies (Peters et al., 2014; Rioult-Pedotti et al., 2000). These results have suggested that layer II/III neurons could participate in motor learning via the synaptic strengthening or weakening rather than via spine formation and elimination (Tjia et al., 2017).

On the other hand, Ma et colleagues reported that spine dynamics of layer II/III apical dendrites in M1 is affected by a single session of treadmill training at a constant moderate speed (8 cm/s) for 40 minutes with a 1-minute break every 5 minutes. Specifically, they compared the rate of spine formation over 24 hours before and after the treadmill training in young mice and reported that mice undergoing treadmill training have an enhanced dendritic spine formation in respect to the untrained mice (***Figure 8A-B***) (Ma et al., 2016).

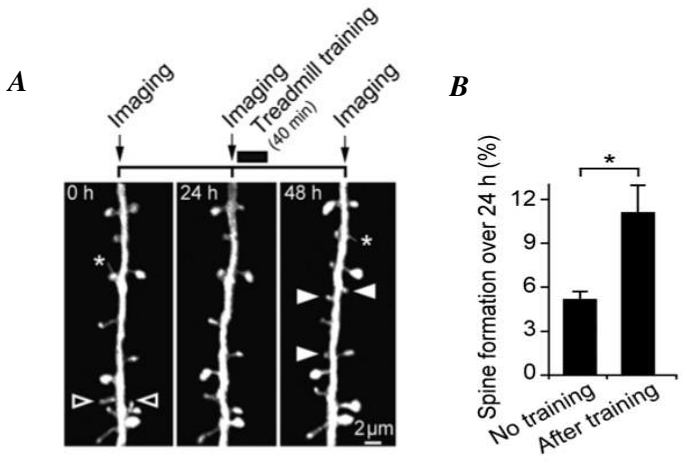

Figure 8. A. Schematic experimental design and representative images showing spine formation and elimination in a dendritic segment labeled with tdTomato. Filled arrows indicate spine formation; opened arrows indicate spine elimination between 0-24h and 24-48 hours. Asterisks mark the filopodia. ***B.*** The rate of dendritic spine formation over 24 hours. It was significantly higher after treadmill training then under non-training conditions. (Images adapted from Ma et al., 2016).

In addition, in the previously discussed study of Yang et colleagues, the increased spines formation in apical dendrites of layer V pyramidal neurons in mice motor cortex was achieved only when mice trained in an accelerated rotarod. Vice versa, when mice walked in a rotarod at constant speed, the apical dendrites did no display changes in spines formation with respect to the untrained mice (Yang

et al., 2009). However, the same author demonstrated that head-fixed treadmill training at constant moderate speed (8 cm/s) for 20 minutes was able to induce spine formation in apical dendrites of layer V pyramidal neurons few years later (Yang et al., 2014).
Thus, moderate motor activity has been demonstrated to affect spines dynamic of layer II/III and layer V of the M1 in mice.

1.3.5 tDCS and M1 plasticity

In 2020, Barbati et colleagues studied the effects of A-tDCS over the M1 in mice physiological conditions. Adult male C57BL/6 mice underwent 3 daily sessions of unilateral A-tDCS for 20 minutes at 250uA, after training in the single pellet reaching task, so once they acquired the motor skill performance. Tested after 24 hours from the end of the tDCS protocol, stimulated mice showed an enhanced success rate in the reaching task in respect to their baseline and compared to the unvaried success rate of the non-stimulated group.
Similarly, mice were also tested for their forelimb grip strength before and 24 hours after the stimulation. The group receiving the A-tDCS displayed a higher grip strength in respect to their baseline and compared to the control group (Barbati et al., 2020). Thus, repeated sessions of A-tDCS over the M1 resulted in an improvement of already acquired motor skills as well as in an enhancement of forelimb strength contralateral to the stimulated M1 region.
In addition to the motor performance, stimulated mice displayed increased LTP and enhanced basal synaptic transmission et layer II/III horizontal connections.
They also found an increased spine density in both apical and basal dendrites of layer II/III pyramidal neurons in the stimulated M1 in comparison to the sham group, 24 hours after the end of tDCS protocol (Barbati et al., 2020).

A-tDCS is known to affect molecular determinants of synaptic plasticity, including immediate early genes (IEG) as c-fos and activity regulated cytoskeleton-associated protein (Arc) (Kim et al., 2017; Ranieri et al., 2012), brain-derived neurotrophic factor (BDNF), phosphorylated CREB (Podda et al., 2016), CaMKII.
It has been reported in rats that c-Fos and Arc mRNA levels were increased in the sensorimotor cortex stimulated with A-tDCS for 20 minutes for 7 consecutive days. In contrast, the contra-lateral mRNA levels were not affected by the tDCS.

Similar results were obtained for the ispi- and contra-lateral mRNA levels of BDNF, phosphorylated CREB, CaMKII (Barbati et al., 2020; Kim et al., 2017).

In humans, tDCS effects on the excitability of contralateral, non-stimulated, motor cortex has been studied at first by Lang et colleagues in 2004. In this study, anodal or cathodal tDCS have been applied for 10 minutes to the left primary motor cortex, resulting in a facilitation or a suppression of MEPs evoked according to the direction of the current. The effects lasted shorter for A-tDCS in respect to cathodal tDCS. In contrast, MEPs evoked from the contralateral motor cortex were not affected. However, the transcallosal inhibition from the right to the left motor cortex did change in duration(Lang et al., 2004). A more recent study deals with the effects of 20 minutes cathodal tDCS on the excitability of stimulated and contralateral motor cortex by using single and paired-pulse TMS approaches in healthy volunteers. The experiment resulted in a significant suppression of cortical excitability in the stimulated hemisphere, with the inhibitory effects lasting for 3 hours after the end of tDCS. However, in agreement with the previous reported study, no change in MEPs amplitudes were found in the contralateral hemisphere (Di Lazzaro et al., 2012).

1.4 tDCS & physical activity: is there a synergistic effect?

As discussed exhaustively in chapter 1.2.4, tDCS produces a change in the membrane potential which is not sufficient to induce spikes in resting cortical pyramidal neurons and the emerging idea is that its outcomes may depend on the state of the brain when the stimulation is applied (Bergmann, 2018; Bikson et al., 2012; Rahman et al., 2017).
Neurons in a stimulated region are interconnected with other ipsi- or/and contra-lateral regions and all together constitute a brain network, which spontaneous activity is regulated by neural rhythmic fluctuations under the control of afferent pathways and cortico-cortical connections (Draguhn & Buzsáki, 2004; Rahman et al., 2017; Sadaghiani et al., 2010; Schroeder & Lakatos, 2009; VanRullen & Koch, 2003). Hence, the local or global network activity state determines the effects of tDCS in a specific region.

Since the afferent inputs can participate to the network activity state, they can influence tDCS effects on both the stimulated region and its interconnected ones.

This concept is broadly analogous to the combination of tDCS with a cognitive or motor task, that can co-activate the targeted network (Gellner et al., 2020; Jackson et al., 2016; Paciello et al., 2018; Wang et al., 2021).

An *in vitro* study of Fritsch et colleagues showed that the potentiation of field excitatory post synaptic potentials (fEPSP) amplitude in layer II/III neurons of M1 mice slices, was elicited only when 15 minutes of anodal DCS was applied concomitantly with a repetitive low-frequency synaptic activation (LFS) of the vertical pathway (layer V – Layer II/III) at the specific frequency of 0.1Hz. If the A-tDCS was combined with LFS at lower or higher frequencies the effect was less effective (Fritsch et al., 2010).

Later, Gellner et colleagues observed that A-tDCS applied over the sensorimotor cortex in combination with the contralateral electrical forepaw stimulation (eFS) rapidly increased the spine density in the apical dendrites of layer V pyramidal neurons in anesthetized mice. However, when tDCS or eFS was applied singularly, no changes in dendritic spine density were noted. Moreover, the combo-induced changes lasted for 24 hours after the stimulation. Specifically, both an increase of the original spine survival and the formation of new spines, morphologically characterized by larger head sizes, were observed (Gellner et al., 2020).

These evidence in animal studies suggest that the afferent input effectively can modulate tDCS effects, as tDCS outcomes are not noted whit this intervention alone, and strongly affirm the brain state dependency of tDCS efficacy.

The idea that tDCS-induced effects may depend on the ongoing activity of the system has recently prompted researchers to combine tDCS with other types of interventions, such as physical activity in humans (Beretta et al., 2020; Wang et al., 2021).

It is already known, in fact, that tDCS significantly enhances physical performance in healthy and stroke people indeed (Tanaka et al., 2009). It is also accepted that both physical exercise and A-tDCS can increase the M1 cortical excitability by MEPs amplitude analysis in humans (Mazzoleni et al., 2019). In reviews articles of 2019 and 2022, it is suggested that the combination of tDCS and physical training induces significant improvement in motor cortex excitability, physical performance and motor learning in healthy subjects (Wang et al., 2021).

The idea that emerges from these papers is that the combination of tDCS with motor activity may play a synergistic role in improving the motor performance, meaning that the coupling could promote benefits to a greater extent on synaptic intensity and brain functional connectivity beyond the effects

of each approach alone (F. Steinberg et al., 2019; Wang et al., 2021). Nevertheless, the mechanisms underlying this synergistic, mixed-type, intervention remain unclear still.

4. Physiological aging & M1 plasticity

Life expectancy has notably grown in the last century. It is widely known that physiological aging leads to a cognitive and physical decline which constrains daily activities and undermines the independence of the elderly population (Grady, 2012).

The decline of motor functions has been observed since the middle age in both humans (between the late 40s and late 50s) and rodents (Inoue & Nishimune, 2023). In fact, behavioral tests in middle-aged mice (between 13 and 19 months old) reported an impairment in balance, motor coordination and gait (Broom et al., 2021; Takahashi et al., 2016). These studies suggest that motor function decline starts early during the lifespan.

Moreover, the mechanism responsible for the motor alterations during the middle-aged stage seems to differ from the one of the advanced age. This difference seems to reside in the fact that in middle-aged mice, the spinal motor neurons are not compromised. Therefore, in this early phase of elderly the decline had been attributed to the motor cortical network (Ghasemian-Shirvan et al., 2020; Inoue & Nishimune, 2023; Tatti et al., 2016).

In the motor cortex, physiological aging has been observed to cause cortical atrophy and cortical excitability and neurotransmitters levels alterations (***Figure 9***) (Inoue & Nishimune, 2023).

Cortical atrophy is associated with structural changes in neurons, such as shrinkage in the soma size, a reduction in dendrites arborization complexity and length and a loss of dendritic spines (Dickstein et al., 2007).

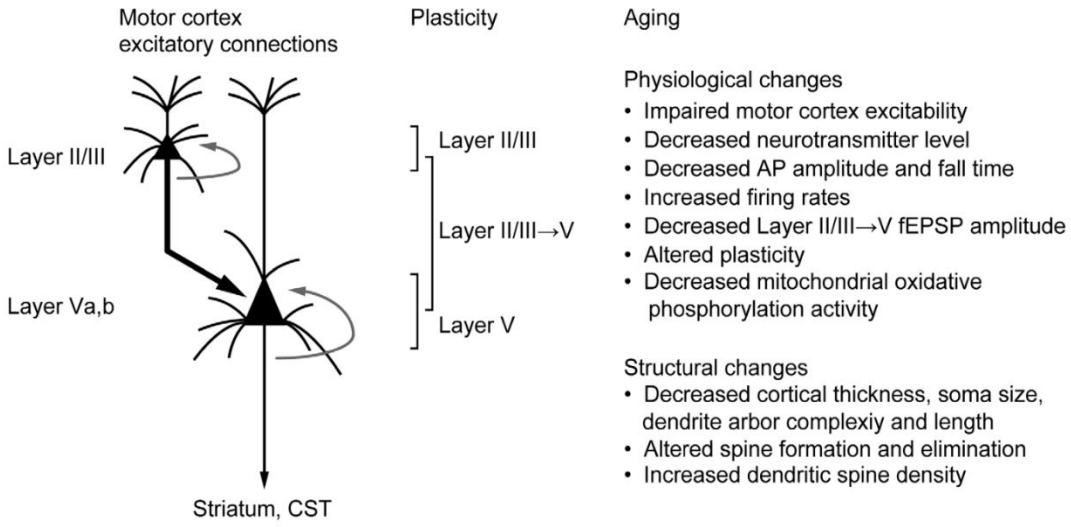

Figure 9. Aged-related physiological and structural changes in motor cortex excitatory connections. The excitatory connection between layer II/III and layer V is the main intralaminar connection in the motor cortex. Grays arrows indicate intralaminar horizontal connections. CST = corticospinal tract; AP = action potential; fEPSP = field excitatory post synaptic potential. (Image adapted from Inoue & Nishimune, 2023).

In rodents, the alterations in motor cortical excitability have been linked to age-related motor function decline, as in human studies. One presumable underlying mechanism is an altered synaptic plasticity, referring to both the functional and structural alterations of synaptic connections in response to external or internal demands (Inoue & Nishimune, 2023).

In rat slices, NMDA receptor-dependent LTP has been shown to decrease with age indeed. In aged mice it has been also reported a plasticity decline, with an increase of the synaptic threshold for the induction of LTP and an enhanced probability for the induction of long-term depression (LTD) (Foster, 2012).

This phenomenon has been partially attributed to the reduction of spine density in aged brains (van der Zee, 2015). However, in a recent study of Davidson et colleagues, the structural plasticity of dendritic spines of apical dendrites of layer V pyramidal neurons was examined in the M1 of young (3-5 months) and aged (20-23 months) mice. The researchers found an increased spine density in the aged group, along with an elevated spine turnover and short-term stabilization of spines. Contrarily, the long-term survival of spines was lower. The authors proposed that this short-term spine stabilization could be a compensatory mechanism for the loss of connections in the long term. Hence, the aged mice cortex may be in a perpetual state of instability and attempted compensation, leading to a suboptimal connectivity too (Davidson et al., 2020).

Beyond aged-dependent alterations at the local level, the functional connectivity of the motor cortex is impaired in aging. In study it has been reported that aged mice (17-18months) have a reduced functional connectivity in the M1, compared to young mice (2-3 months). Interestingly, most of the

functional connectivity changes were observed in the inter-hemispheric connections. Aged mice showed less inter-hemispheric homotopic functional connectivity of the motor cortex indeed (Albertson et al., 2022).
Despite the increasing data reporting the physiological effects of aging on plasticity and network connectivity, the impact of aging has not been completely clarified.

Additionally, another poorly explored field is how tDCS-induced plasticity can be affected by age and vice versa.
In a recent human study, anodal, cathodal and sham tDCS were applied over the M1 of young (18-30 years), pre-elderly (50-65 years) and elderly (66-80 years) healthy subjects. The cortical excitability was monitored by single-pulse TMS, as index of plasticity. The A-tDCS results showed a significant enhancement in motor cortical excitability in young and pre-elderly groups, compared to sham group. However, no differences were observed in the elder group. On the contrary, cathodal tDCS showed a significant decrease of cortical excitability in all the three groups. Hence, it has been proposed that there is an age-dependent decline of tDCS-induced plasticity effects (Ghasemian-Shirvan et al., 2020), even if animal studies explaining the mechanisms are still missing.

Lastly, it has been proposed that the combo between tDCS and motor activity may be able to enhance motor functioning in the elderly. This hypothesis raised from the human study Hummel et colleagues, indicating that a single session of A-tDCS over the left M1 improve the hand performance in the Jebsen-Taylor hand function test in elder subjects more than in the younger ones. The authors explained this outcome with the fact that older subjects were no more able to reach ceiling performances compared to the young group (Hummel et al., 2010). Later, Parikh and Cole effectively tested the combo between A-tDCS and physical training on the motor performance of dexterous grasping and manipulation in healthy older adults. They obtained that the combo helps older adults to retain their improved performance, contrarily to the group receiving just the motor training (Parikh & Cole, 2014).
In animals the combination of tDCS and physical training have never been applied to elderly, so the underlying mechanisms of actions are still unveiled.

2. AIM OF THE STUDY

Although the enhancing effects of both tDCS and physical activity on motor excitability are well known, only recently the two approaches have been combined. In humans, it has emerged that the coupling acts in a synergistic manner, with addictive effects in respect to the mere summation of the single interventions. However, the mechanisms responsible of the synergistic effects are not clear yet and animal studies on the coupling are still limited.

In rodents, it has been already proved that monolateral A-tDCS alone acts on cell activation and plasticity of the ipsi-lateral M1, however, the effects of the combo A-tDCS-physical activity on plasticity have not been studied. Moreover, motor cortex functional connectivity is also affected by tDCS and the effects of the coupling on this may be important both in physiological and in pre-clinical fields.

For these reasons, the main goal of this study is to evaluate the plasticity effects of combining monolateral A-tDCS and moderate physical activity in the stimulated and non-stimulated motor cortices in young mice.

Of note, physiological aging has been associated to a reduction of M1 plasticity and to changes in tDCS-induced plasticity effects. Thus, the second aim of this investigation is to verify the eventual synergistic effects in elderly.

3. MATERIALS & METHODS

Animals & Experimental design

A total number of 64 male C57BL/6 mice of 2-3 months and 15 ± 2 months were used for this study. The animals were group housed under a 12:12h light/dark cycle at controlled temperature, with free access to food and water.

In this study we investigated the effects of the monolateral A-tDCS on the stimulated (right) and non-stimulated (left) M1 when the stimulation is applied alone or in combination with a moderate physical activity (rotarod at 9 rpm). In a first group of young adult mice, we analyzed the effects of the coupling on cell activation and structural plasticity, by counting cFos+ cells and the dendritic spine density. To this aim, we performed on different slices of the same brain cFos immunofluorescent staining and Golgi-Cox technique, respectively. Then, in another cohort of young animals, we analyzed the LFP M1-M1 coherence, by implanting two electrodes in the right and left M1 and the reference electrode on the cerebellum, to verify the eventual functional connectivity changes in response to the coupling. Additionally, the same analysis for cell activation and structural plasticity were performed on aged mice to investigate if the effects of the combo are maintained in elderly.

At both ages, mice were randomly assigned to one of the following experimental groups, according to the stimulation protocol (sham vs A-tDCS) and physical activity (home cage vs rotarod):

1) Sham/r-, which received sham stimulation in their home cage,
2) Sham/r+, sham treated mice performing rotarod,
3) A-tDCS/r-, A-tDCS mice while in their home-cage,
4) A-tDCS/r+, stimulated mice while walking on the rotarod.

After surgery, mice were single housed for the rest of the experiment. All animal procedures were performed in accordance with the EU Council Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Italian Ministry of Health.

Electrode implantation & tDCS protocol

Mice were anaesthetized by an intraperitoneal injection of ketamine-xylazine (80 mg/kg and 5 mg/kg, respectively) and placed on the stereotaxic frame. Then, the scalp and underlying tissues were removed, and a tubular plastic jack (inner area 4.5 mm^2) was dental cemented over the right M1 area, 2 mm lateral to Bregma (Cambiaghi et al., 2020). During the surgical procedure, the temperature of mice was maintained at 37°C with heating paddles. After surgery, all animals were allowed to recover for 3-4 days.

Before entering the stimulation procedure, all mice started a 9-days habituation to the rotarod apparatus.

The mice were then randomly divided into one of the groups. To deliver A-tDCS, the active electrode was filled with saline solution (0.9% NaCl) and the counter electrode consisted in a saline-soaked sponge, applied over the ventral thorax by using a rubber-made corset.

The stimulation was applied through a homemade battery-driven constant current stimulator, at a current intensity of 240 µA for 10 minutes twice a day with 3 hours break in between, for two consecutive days (***Figure 10A-C***). To avoid a stimulation break effect, the current intensity was ramped instead of switching it on and off immediately. Sham groups of animals underwent the same protocol but no current was delivered. Sham/r- and A-tDCS/r- mice remained in their home cage, while Sham/r+ and A-tDCS/r+ underwent sham or A-tDCS with the rotarod speed maintained constant at 9 rpm.

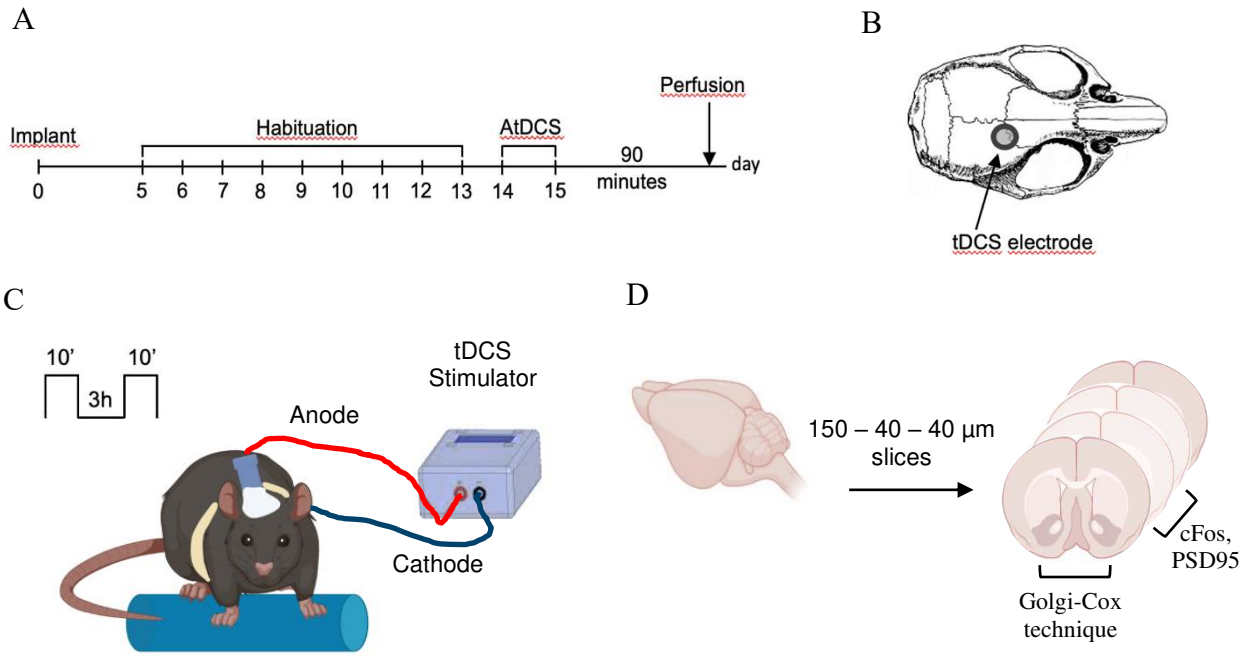

Figure 10. Experimental design & stimulation procedure. ***A.*** Schematic timeline. After the electrodes implant, mice underwent a habituation period of 9 days before the 2 stimulation days. After 90 minutes from the end of the last stimulation, mice were perfused. ***B.*** Representative image of tDCS active electrode location (2mm lateral to Bregma). ***C.*** Representative image of A-tDCS/r+ condition. The mouse is walking on a rotarod while A-tDCS is applied. The protocol consists of 10 minutes, 3 hours break and other 10 minutes for two consecutive days. ***D.*** After the dissection, the brain was cut in sections according to a 150µm-40µm-40µm pattern to allow different histological processes.

Histological Processing

After 90 minutes from the end of the last stimulation, mice were anesthetized perfused transcardially with 0.1 M phosphate buffer solution (PBS), followed by 20 mL of 4% paraformaldehyde. Collected brains were post-fixed for an overnight and stored at 4°C in PBS1X.
Serial coronal sections containing the M1 were cut using a vibratome (Leica VT1200), with a repetitive thickness pattern of 150µm-40µm-40µm (***Figure 10D)***.

For each animal, three 40µm sections within M1 (+1.94, + 1.50, + 1.10 from Bregma) were processed for a free-floating immunofluorescence staining to evaluate cell activation with the early-gene marker cFos (1:200, Synaptic Systems anti-rabbit, cat. 226003).
In a 24well, slices were soaked in the blocking buffer (composed of 2% BSA, 2% goat serum, 0.2% Triton100X in PBS) for 30 minutes, at RT. Then, the solution was substituted with the blocking buffer containing the primary antibody for an overnight, at 4°C. After 3 washes in PBS for 10 minutes each, sections were incubated with the secondary antibody anti-rabbit 594 (1:1000, Sigma cat. SAB4600107) for 2h at room temperature. Staining with DAPI (1:5000 in PBS) for 6 minutes followed other three washes in PBS. Successively, after a last PBS wash of 10 minutes, slides were mounted with DAKO Fluorescence mounting medium (DAKO).
Images of layer II/III and layer V of the left and right M1 were acquired by using Leica Fluorescence Microscope (Leica Systems, Wetzlar, Germany) with a 20X objective lens. The images were 1392x1040 of pixels size and they constituted the Region of Interests (ROIs) used to analyze the number of activated cells. In fact, cFos stained cells were manually counted by an operator who was unware of the experimental group through the plugin 'Cell Counter' of ImageJ Software.

To evaluate the post-synaptic density, an on-slide immunofluorescence staining marking the scaffolding protein PSD95 was performed. M1 sections of 40µm were mounted on slides and let them dry. An antigen retrieval protocol was used for the antigen unmasking. Briefly, slides were soaked in a staining jar containing 10mM of sodium citrate solution and boiled with microwave (600W, 3timesx5minutes). Sections were then covered with the permeabilization solution (0,4% Triton100X in PBS) for 40 minutes, followed with the blocking solution (0,4% Triton100X, 2% BSA, 2% NGS in PBS) with the primary antibody anti-PSD95 (1:400, GeneTex anti-rabbit, cat. GTX13309) for an overnight at +4°C. After 3 washes in PBS for 15 minutes each, sections were incubated with the secondary antibody anti-rabbit 647 (1:1000, Sigma cat. SAB4600184) for 2h at room temperature. Other three washes preceded the staining with DAPI for 6 minutes and a last PBS wash. Finally, sections were mounted with DAKO Fluorescence mounting medium (DAKO).

To analyze PSD95 signal, two z-stacks images (1024x1024 pixels, step size 0.5 µm, z-stack thickness 5µm) of layer II/III and layer V of left and right M1 were acquired with Leica-Sp5 Confocal microscope with a 63X glycerol immersion objective at 1.5 zoom. Later, two ROIs from each image were randomly selected (288x266 pixels size) and the percentage of area fraction occupied by the PSD95 signal was calculated through the ImageJ Software.

For the dendritic spine density analysis, 150 µm slices were processed for Golgi-Cox staining. Two M1 sections/animal were immersed in 1mL of Golgi-Cox solution, made by mixing Solution A and B of Rapid Golgi-Cox Staining Kit. After 14 days, free-floating sections received a 5-minute wash in running water and then they were treated firstly with Ilfosol3 (Ilford, cat. 1131778; 1Ilfosol + 9 H2O parts) for 6 minutes and then with Rapid Fixer (Ilford, cat. 1984253; 1Rapid Fixer + 9H2O parts) for 5 minutes. A 5-minute wash in H20 was used to stop the Rapid Fixer action on the tissue. After being dehydrated with 50%, 70%, 80% and 96% of ethanol for 1 minute each, slices were mounted with Eukitt Quick-hardening mounting medium (Sigma-Aldrich, cat. 25608-33-7). Dendritic spines of both right and left M1 cortical pyramidal neurons were manually counted by using Leica Fluorescence Microscope (Leica Systems, Wetzlar, Germany) with a 100X oil-immersed objective lens in brightfield. In layer II/III we analyzed the 5-6 distal segments of tertiary apical and secondary basal dendrites. In layer V we investigated 5-6 secondary basal dendrites and tertiary apical dendrites extending in layer II/III and layer V. The dendritic spine density was calculated by dividing the number of spines over the dendritic length in µm.

In vivo LFP recordings & analysis

The recordings of extracellular field potentials were made in awake mice, while resting, at baseline (day 8, pre-stimulation) and after the stimulation protocol (day 15, post-stimulation) (***Figure 11A, E***). LFP was recorded with tungsten wires (∅ 50 µm) implanted in the right and left M1 cortex, according to the following coordinates: AP = +0.8 (1.1) mm, ML = ±1.6 (1.5) mm from Bregma and DV = -0.5 mm from the brain surface, with the reference electrode over the cerebellum.
Briefly, mice were anesthetized with a cocktail of ketamine-xylazine (80mg/Kg and 5mg/Kg, respectively, i.p.) and placed on a stereotaxic frame. Once the skull was incised, small cranial holes were drilled according to the previously described coordinates, to allow the insertion of the LFP electrodes and reference and dental cement was used to fix them. Finally, the tDCS plastic tubular jack (inner area 4.5 mm^2) was cemented behind the right implanted electrode.
To verify the location of the electrodes Cresyl Violet staining was performed (***Figure 11B-E***).

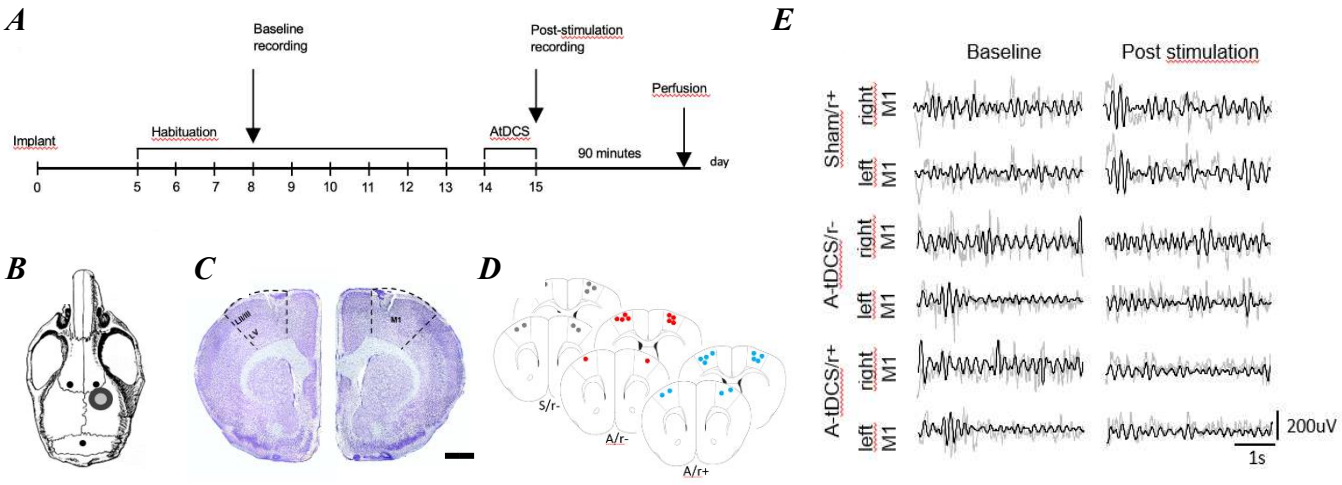

Figure 11. LFP recordings & analysis. ***A.*** Schematic timeline indicating the pre- and post- stimulation LFP recording during the tDCS protocol. The pre-stimulation recording is on day 8, the post-stimulation recording on day 15, after the end of the last stimulation. ***B.*** Schematic brain image depicting the location of the implanted M1 electrodes and of the cerebellar reference electrode (in black). In gray the placement of the plastic tubular jack for tDCS, with the dark grey external circle indicating the thickness of the tube. ***C.*** Representative image of Nissl staining for the localization of the electrodes. The electrodes are placed in the right and left M1. Scale bar = 1mm. ***D***. Schematic images of the electrodes' location in sections from +1.50 and +1.10mm from Bregma. ***E.*** Representative 4-seconds traces of the LFP baseline and post-stimulation recordings in the right and left M1 of the three conditions: Sham/r+, A-tDCS/r- and A-tDCS/r+. In grey the row LFP trace, in black the overlayed 4-8 Hz filtered trace.

All the recordings lasted 1 to 3 minutes and were performed in a customized Faraday chamber, in which the rotarod was placed. The Software Galileo NT (EBNeuro) was used to record the LFP traces, with two channels belonging to the left and to the right M1 activity.

A customized Python script was used for offline coherence analysis. For each animal, three 2-seconds epochs were averaged both for the baseline and for the post-stimulation recordings. As in Cambiaghi et. al. (Cambiaghi et al., 2016), differences in coherence were obtained by subtracting the mean coherence values (post-stimulation – pre-stimulation) and measured within the following frequency bands: delta (0.5-3.5 Hz), theta1 (4-8 Hz), theta2 (8.5-12 Hz), alpha (12.5-15 Hz) and gamma (15.5-20 Hz). Mice mean coherence values were averaged within the same group condition.

Statistical Analysis

Statistical analysis was performed using Prism 8.3 Software (GraphPad, La Jolla, CA, USA).

After normality distribution testing (Shapiro-Wilk test), inter-hemispheric and inter-groups data were analyzed with ordinary one-way ANOVA with Tukey's or Dunn's multiple comparisons test, or with two-tailed unpaired-t test. Differences among groups were considered statistically significant with a $p<0.05$.

4. RESULTS

In humans, tDCS combined with physical training led to enhances motor cortex excitability, physical performance and motor learning (Wang et al., 2021). This evidence indicates that tDCS effects depend on the concurrent neural activity, so motor cortex state (i.e. excitability) must be considered when applying this stimulation (Bergmann, 2018). In the sliced mouse motor cortex, anodal DCS coupled with synaptic activation shows a promotion of long-lasting synaptic plasticity, not observed by DCS alone (Fritsch et al., 2010). Similarly, in anesthetized and behaving mice, A-tDCS applied during electrical or physiological activation of the stimulated motor cortex (i.e 0.1 Hz forepaw stimulation and locomotion, respectively) result in enhanced motor learning and structural synaptic plasticity (Gellner et al., 2020). Of note, inter-hemispheric communication between motor cortices is essential for a simple motor behavior, such as locomotion (Lopes et al., 2023).

Hence, we asked how monolateral tDCS applied during walking affect the non-stimulated contralateral motor cortex. To this aim, we applied A-tDCS over the right mouse M1 during moderate locomotion or home-cage activity for two consecutive days and we examined both hemispheres activation, functional interaction, and dendritic spine modulation.

Coupling tDCS and physical activity enhances motor cortex activation

Initially, we tested how monolateral A-tDCS activates the contralateral M1 activity when combined with its physiological activation (i.e. walking). In the stimulated (right) and non-stimulated (left) M1 (***Figure 12A***), we tested cFos levels, as a measure of cell activation, in layer II/III and in layer V while mice were walking or at rest (***Figure 12B***). Since motor activity is known to induce cFos expression in a subset of M1 neurons (Kleim et al., 1996), in 2-3 months-old mice we first examined the effects of walking by comparing non-stimulated mice in the home-cage (Sham/r-) with the ones walking on the rotarod (Sham/r+). (***Figure 12C-F***). In both layer II/III and V, physical activity resulted in an increased number of cFos+ cells ($p=0.04$, unpaired t-test, and $p=0.0019$, Mann-Whitney test, respectively), when the values of left and right hemisphere of both Sham/r- and Sham/r+ conditions are unified. When applied in both humans and animals, monolateral M1 A-tDCS enhances neural activity mainly in the stimulated hemisphere, as evidenced by MEPs or IEGs (Cambiaghi et al., 2010; Kim et al., 2017; Nitsche & Paulus, 2000), however, tDCS has been also observed to activate the expression of cFos both beneath the electrode as also in interconnected regions (Cambiaghi et al., 2020).

In this study, our approach has been observed to affect tDCS effects in layer II/III (Two-way ANOVA followed by Tukey's post hoc correction. Treatment x hemisphere interaction: $F_{3,24} = 21.25$, $p < 0.0001$; treatment factor: $F_{3,24} = 137{,}9$, $p < 0.0001$; hemisphere factor: $F_{1,24} = 54.64$, $p < 0.0001$) and

in layer V (Two-way ANOVA followed by Tukey's post hoc correction. Treatment x hemisphere interaction: $F_{3,24}$ =17.68, $p < 0.0001$; treatment factor: $F_{3,24}$ = 111.3, $p < 0.0001$; hemisphere factor: $F_{1,24}$ = 49, $p < 0.0001$).

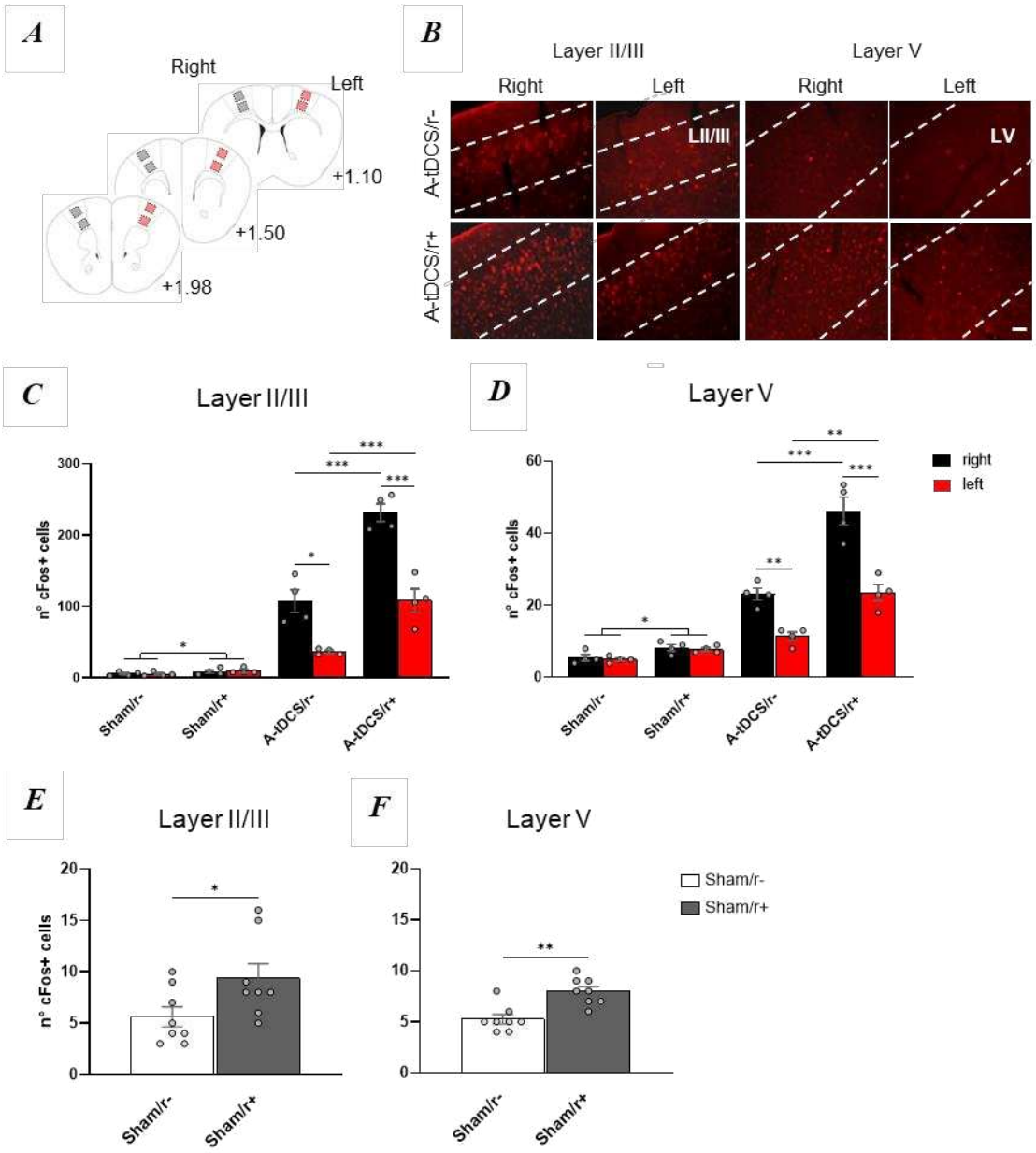

Figure 12. Coupling tDCS and physical activity enhances motor cortex activation. ***A.*** Schematic representation of the Regions of interest (ROIs) used to count cFos+ cells. In three sections (+1.98; + 1.50; +1.10 mm from Bregma), images of layer II/III and layer V were acquired in both left and right M1. ***B.*** Representative images of cFos+ cells (in red) in A-tDCS/r- and A-tDCS/r+ experimental conditions, in both left and right hemispheres. Images acquired at 20X objective with the fluorescence microscope. The dotted white lines define M1 layer II/III and layer V. Scale bar = 20µm. ***C-D.*** The number of cFos+ cells in M1 layer II/III (***C***) and in layer V (***D***) in the non-stimulated (Sham/r- and Sham/r+) and the stimulated (A-tDCS/r- and A-tDCS/r+) conditions (n = 4/ group). ***E-F.*** The number of cFos+ cells in the two non-stimulated conditions

when left and right hemisphere values are unified in layer II/III (***E***) and layer V (***F***) (n = 8 /group). Gray dots indicate individual values. Data are expressed as mean ± SEM. Analysis with Two-way ANOVA followed by Tukey's Post hoc correction (graphs A, B), unpaired t-test (graphs B, D). * p<0.05; ** p<0.01; *** p<0.001.

Here we found that monolateral M1 stimulation in resting mice (A-tDCS/r-) had increased cell activation of the ipsilateral (i.e. right) with respect to the contralateral M1 in layer II/III and V (*p=0.0003* and *p=0.002*, respectively) (***Figure 12C, D***). Then, we considered the synergistic effects of A-tDCS and motor activity (A-tDCS/r+), a protocol known to improve motor cortex functionality compared to tDCS-or-exercise/training-only intervention in humans (Wang et al., 2021). In this group, we observed higher cell activation in the stimulated hemisphere when compared to A-tDCS/r- in both layer II/III (*p<0.0001*) (***Figure 12C***) and layer V (*p<0.0001*) (***Figure 12D***), demonstrating that the brain state represents a key aspect for tDCS effects. Interestingly, while remaining a significant interhemispheric difference in A-tDCS/r+ in both layers (*p<0.0001*), the non-stimulated M1 in the A-tDCS/r+ showed a higher activation with respect to the non-stimulated M1 in the A-tDCS/r- in both layers (*p=0.0003* and *p=0.0016*, respectively) (***Figure 12C, D***), suggesting an indirect enhancing effect of tDCS. This outcome prompted us to explore whether the bilateral M1 enhancement might be associated with interhemispheric functional changes.

Combining tDCS and physical activity enhances the communication between the motor cortices

Thus, we investigated M1-M1 connectivity by measuring LFP coherence, as an index of interhemispheric synchronous activity. Baseline M1-M1 coherence was recorded immediately after the first day of walking on the rotarod (day 8) and compared to the post-stimulation recording, executed immediately after the last tDCS session (day 15) (***Figure 11A***). For each condition, post-stimulation − baseline M1-M1 synchrony (Δcoherence) was compared among groups for each frequency band (Two-way ANOVA followed by Dunn's post hoc correction. Groups x frequencies interaction: $F_{80,574} = 0.7711$, *p=0,9260*; frequencies factor: $F_{40,574} = 0.7751$, *p = 0.8396*; groups factor: $F_{2,574} = 4.010$, *p = 0.0187*) (***Figure 13A-D***). This analysis did not report any difference between Sham/r+ and A-tDCS/r- mice for each frequency band, while A-tDCS/r+ mice revealed increased coherence levels in the low-theta band (4-8 Hz), (*p=0.02*, Kruskal-Wallis test), but not in the other frequency ranges (***Figure 13E-F***). These results suggest that, when combining tDCS and physical activity, motor cortex is more synchronized in the theta frequency, which characterizes motor network connectivity (Noga et al., 2017).

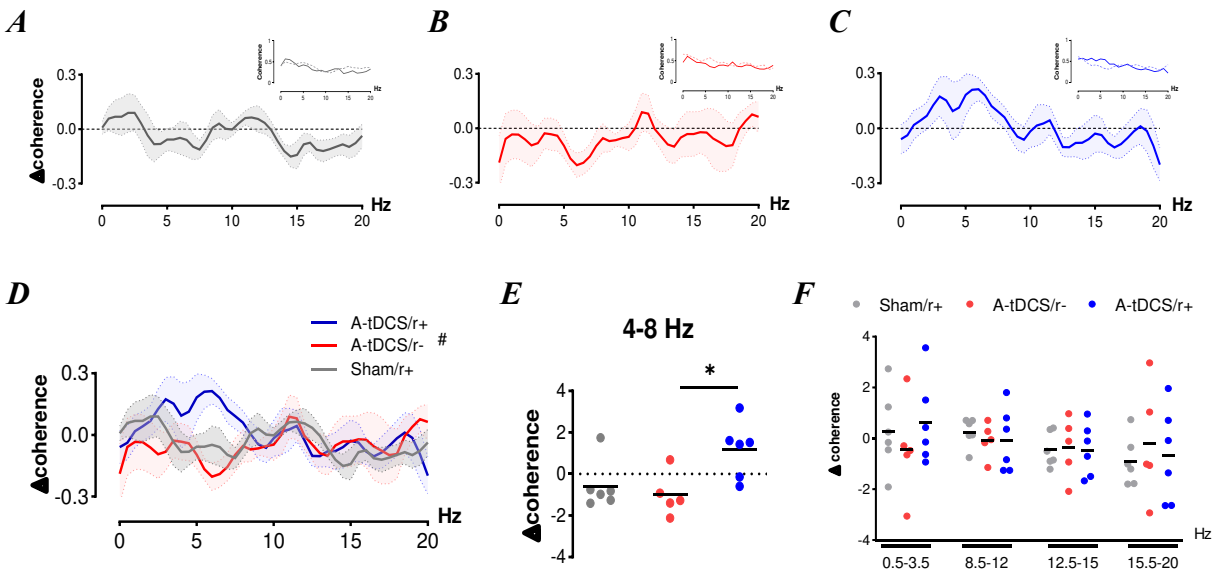

Figure 13. Combining tDCS and physical activity enhances M1-M1 communication. ***A-C.*** Δcoherence (post-stimulation – baseline) M1-M1 synchrony in Sham/r- (***A***), A-tDCS/r- (***B***) and A-tDCS/r+ (***C***) according to the frequencies (Hz). Filled lines indicate the mean; coloured areas represent the respective SEM. In the small graphs above, the dotted line refers to the baseline mean values and the filled line to the post-stimulation mean values for each condition. S/r+ n= 6; A/r- n=5; A/r+ n=6. ***D.*** Graph plotting the Δcoherence of all the three conditions up to 20 Hz. Analysis two-way ANOVA, with Dunn's multiple comparisons. * p<0.05. ***E.*** The Δcoherence is significantly higher in the theta frequency band (4-8 Hz), but only when tDCS and physical activity are combined. Each dot indicates an animal, black lines indicate the median values. ***F.*** Δcoherence in the other frequency bands for the three conditions. Data are expressed as mean ± SEM. Analysis with Kruskal-Wallis test. * p<0.05.

tDCS combined with a physiological motor cortex activation boost dendritic spine density in both hemispheres

Structural plasticity, such as the dynamic changes in spine number, is associated to experience-dependent changes in neural circuits. Dendritic spines represent the postsynaptic partner of most excitatory input received by M1 neuron through intra- and interhemispheric connections and dendritic spine density are known to play an important role in the formation and modulation of neural circuit functionality (Yu & Zuo, 2011).

In our mice, physical activity did not result in increased M1 mean spine density both in layer II/III and in layer V (***Figure 14A-B***). These findings are in line with similar data in which mice were subject to slowly constant speed rotarod (Yang et al., 2009).

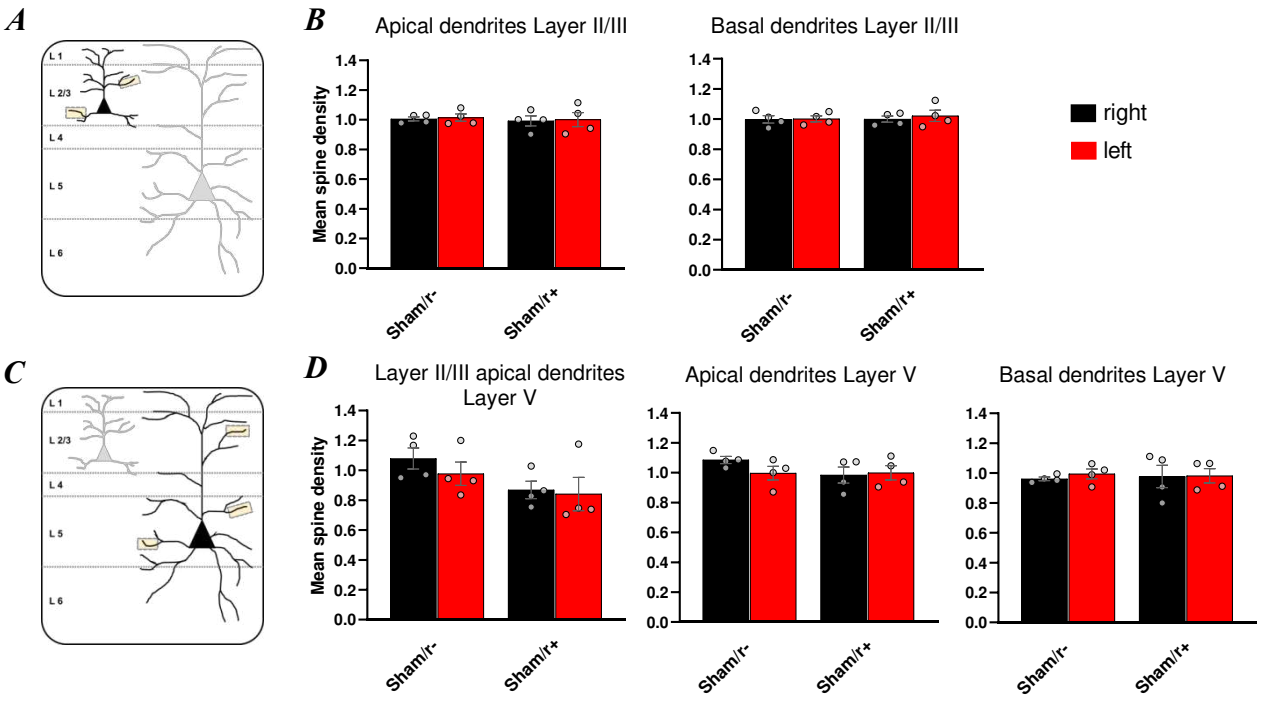

Figure 14. Dendritic spine density in Sham/r- and Sham/r+ groups in young adults. A. Schematic representation of the dendritic segments considered for the dendritic spine density analysis in layer II/III pyramidal neurons. ***B.*** The mean spine density in the distal apical (on the left) and basal (on the right) dendrites of layer II/III pyramidal neurons. ***C.*** Schematic representation of the dendritic segments considered for the dendritic spine density analysis in layer V pyramidal neurons. ***D.*** The mean spine density in layer II/III apical (on the left), layer V apical (in the middle) and basal (on the right) dendrites of M1 layer V pyramidal neurons. Gray dots indicate individual values (n=4/group). Data are expressed as mean ± SEM. Analysis with One-way ANOVA followed by Tukey's Post hoc correction.

On the contrary, A-tDCS increased the mean spine density in layer II/III apical (One-way ANOVA followed by Tukey's post hoc correction, $F_{3,12} = 5.779$, *p=0.04*) and basal dendrites ($F_{3,12} = 8.659$, *p=0.004*) (***Figure* 15A-C**), similarly to what already observed after 3 sessions of tDCS in the awake mouse in layer II/III apical and basal dendrites (Barbati et al., 2020).

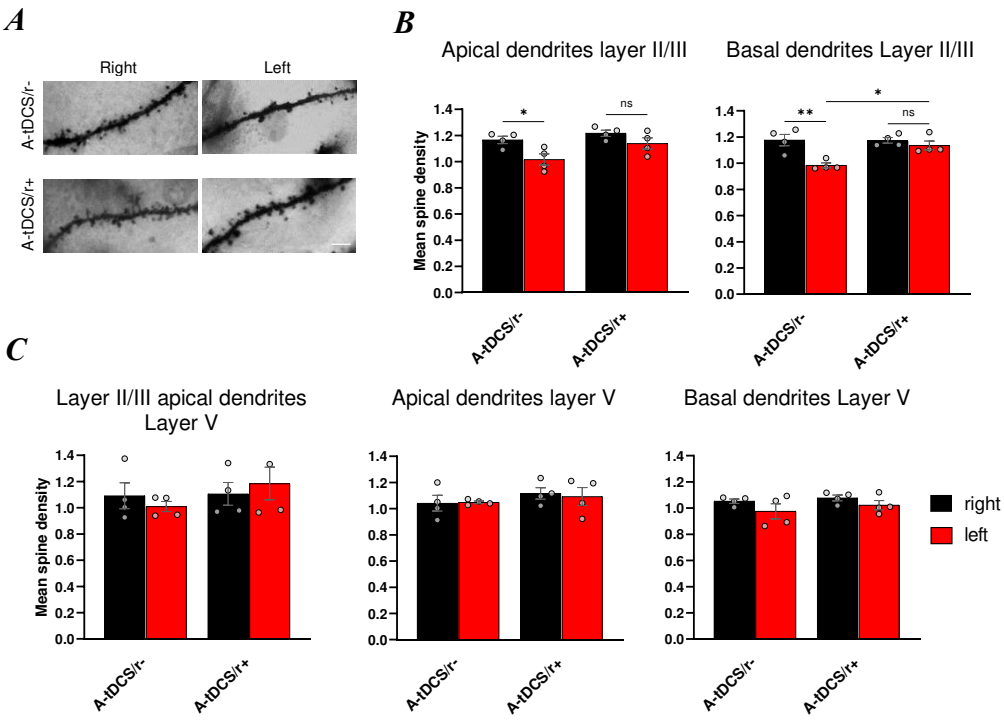

Figure 15. tDCS combined with the physiological motor cortex activation boost dendritic spine density in both hemispheres in layer II/III. A. Representative images of layer II/III apical dendrites in right and left M1, in A-tDCS/r- and A-tDCS/r+ conditions. Images acquired at 100X oil immersed brightfield microscope, with Neurolucida Software. Scale bar = 5µm. ***B.*** The mean spine density in the distal apical (on the left) and basal (on the right) dendrites of layer II/III pyramidal neurons. ***C.*** The mean spine density in layer II/III apical (on the left), layer V apical (in the middle) and basal (on the right) dendrites of layer V M1 pyramidal neurons. Gray dots indicate individual values (n=4/group). Data are expressed as mean ± SEM. Analysis with one-way ANOVA followed by Tukey's Post hoc correction. * $p<0.05$; ** $p<0.01$.

Though not increasing spine density alone, walking activity in combination with A-tDCS resulted in a larger number of spines in the entire M1, both basal and apical dendrites. Moreover, in basal dendrites, the A-tDCS/r+ unstimulated M1 had higher spine density with respect to the A-tDCS/r- unstimulated cortex ($F_{3,12}$ = 8.659, *p=0.02*; ***Figure 15B***). Taken together these data indicate an indirect boosting effect of A-tDCS when combined with physiological increase of M1 excitability. When analyzing the layer V, no effects of stimulation and/or walking was found (***Figure 15C***), analogous to what observed in anesthetized animals (Gellner et al., 2020).

The post-synaptic scaffolding protein PSD-95 exerts a key role in the stabilization of excitatory synapses and spines (Ehrlich et al., 2007). To measure to what extent the increased spine density is related to a synaptic potentiation when A-tDCS is applied during different brain states, in A-tDCS/r-

and A-tDCS/r+ we measured the percentage of PSD-95+ fraction area in M1 (***Figure 16A***). For both conditions, PSD-95 signal was increased in the right M1 in layer II/III, with a significantly lower percentage only in the unstimulated side (i.e. left) in the A-tDCS/r- condition (*p=0.04*, unpaired t-test) while A-tDCS/r+ showed similar levels of PSD-95 expression (***Figure 16B***). As for spine data, in layer V PDS-95+ area did not differ between conditions (***Figure 16C***).

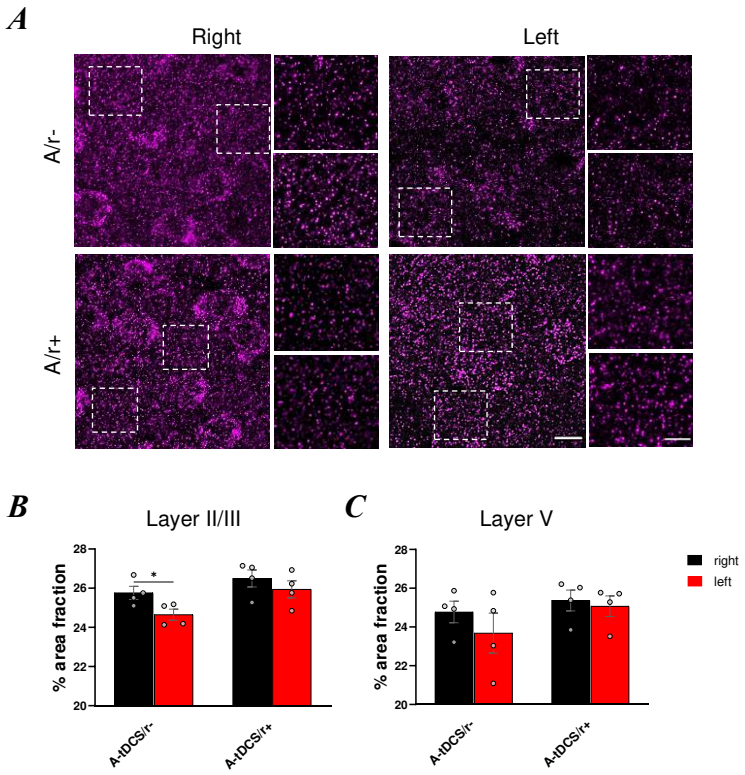

Figure 16. tDCS combined with a physiological motor cortex activation increases the percentage of PSD95+ area fraction in layer II/III. ***A.*** Representative confocal images at 63X magnification of right and left M1 in layer II/III, stained with anti-PSD95 antibody (in magenta) in A-tDCS/r- and A-tDCS/r+ conditions (scale bar = 10µm). The dotted squares represent the ROIs, zoomed in on the right (scale bar = 2µm). ***B-C***. The % of area fraction of right and left layer II/III ***(B)*** and of layer V ***(C)*** M1 in A-tDCS/r- and A-tDCS/r+ conditions. Grey dots indicate individual values (n=4 per group). Data are expressed as mean ± SEM. Analysis with unpaired-t test. * $p<0.05$.

The effects of the coupling in physiological aging

Finally, we asked whether the impact of brain state activity on tDCS aftereffects observed in young mice was also present while normal aging. Indeed, several neurobiological changes are known to happen during lifespan, and tDCS may represent a great opportunity for healthy elderly, identified as a promising target population (Habich et al., 2020). To this aim, we tested our combined protocol in healthy aging mice, ranging 15±2 months.

Analysis of cFos+ cells in layer II/III showed a cell activation similar to young adult mice. The comparison between Sham/r- and Sham/r+ mice showed a significant increase in the number of cFos+ cells in the Sham/r+ group ($p = 0.006$, unpaired t-test), when the left and right hemisphere of Sham/r- and Sham/r+ conditions are unified (***Figure 17A, C)***.

Even in elderly mice, our approach has affected tDCS effects in layer II/III (Two-way ANOVA followed by Tukey's post hoc correction. Treatment x hemisphere interaction: $F_{3,24} = 5.936$, $p = 0.0035$; treatment factor: $F_{3,24} = 59.9$, $p < 0.0001$; hemisphere factor: $F_{1,24} = 16.92$, $p = 0.0004$) and in layer V (Two-way ANOVA followed by Tukey's post hoc correction. Treatment x hemisphere interaction: $F_{3,24} = 3.320$, $p = 0.0037$; treatment factor: $F_{3,24} = 18.34$, $p < 0.0001$; hemisphere factor: $F_{1,24} = 9.706$, $p = 0.0047$)

In layer II/III, we found that monolateral M1 stimulation in resting mice (A-tDCS/r-) resulted in increased cell activation of the ipsilateral (i.e. right) with respect to the contralateral M1, as in young adults ($p=0.01$) (***Figure 17A)***. When considered the synergistic effects of A-tDCS and motor activity (A-tDCS/r+), in this group we observed higher cell activation in the stimulated hemisphere when compared to A-tDCS/r-, in layer II/III ($p=0.01$). In layer II/III while remaining a significant interhemispheric difference in A-tDCS/r+ ($p=0.004$), the non-stimulated M1 in the A-tDCS/r+ showed a higher activation with respect to the non-stimulated M1 in the A-tDCS/r- ($p=0.03$) (***Figure 17A***), suggesting an indirect enhancing effect of tDCS. In layer V, together with no effects in walking (***Figure 17D),*** we observed we observed an A-tDCS effects in the A-tDCS/r+ condition ($p=0.03$) but not the in the A/r- (***Figure 17B***).

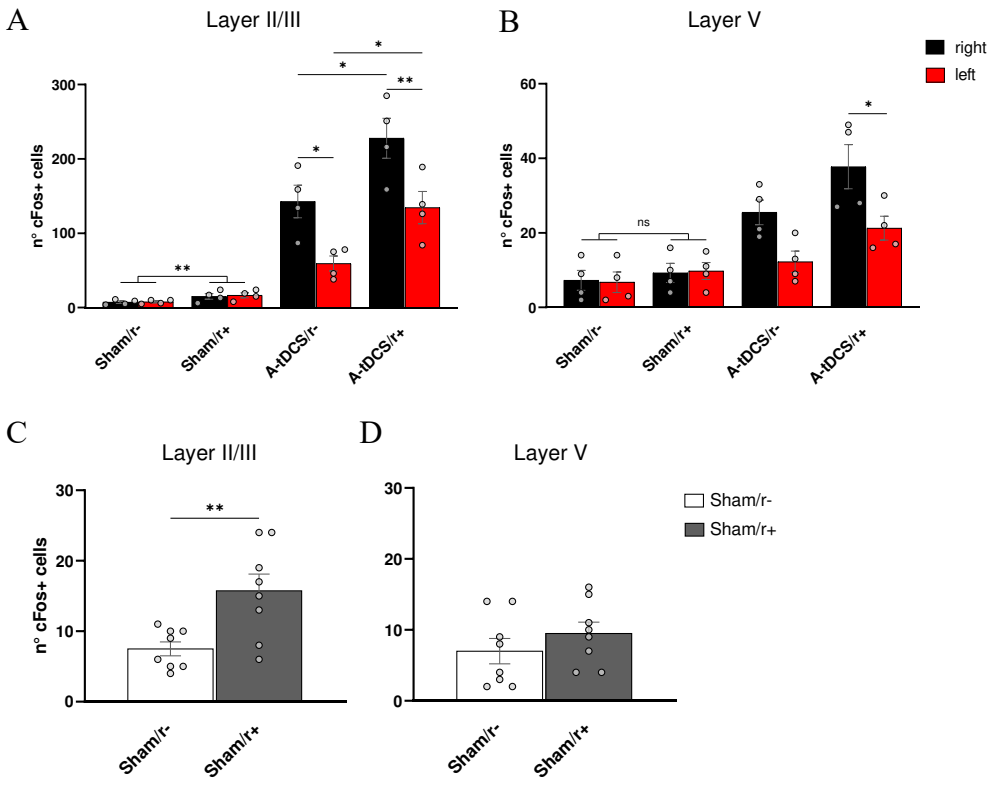

Figure 17. ***Coupling tDCS and physical activity enhances motor cortex activation in aged mice. A-B.*** The number of cFos+ cells in M1 layer II/III (***A***) and in layer V (***B***) in the non-stimulated (Sham/r- and Sham/r+) and the stimulated (A-tDCS/r- and A-tDCS/r+) conditions (n = 4/ group). ***C-D.*** The number of cFos+ cells in the two non-stimulated conditions when left and right hemisphere values are unified in layer II/III (***C***) and layer V (***D***) (n = 8 /group).

Gray dots indicate individual values. Data are expressed as mean ± SEM. Analysis with Two-way ANOVA followed by Tukey's Post hoc correction (graphs A, B), unpaired t-test (graphs B, D). * $p<0.05$; ** $p<0.01$.

Though the initial spine density was slightly lower than young mice (***Figure 18A-B)***, dendritic spine density in aging mice reflects the achievement observed in young animals, in both layer II/III and V. In our mice, physical activity did not result in increased M1 mean spine density both in layer II/III and in layer V.

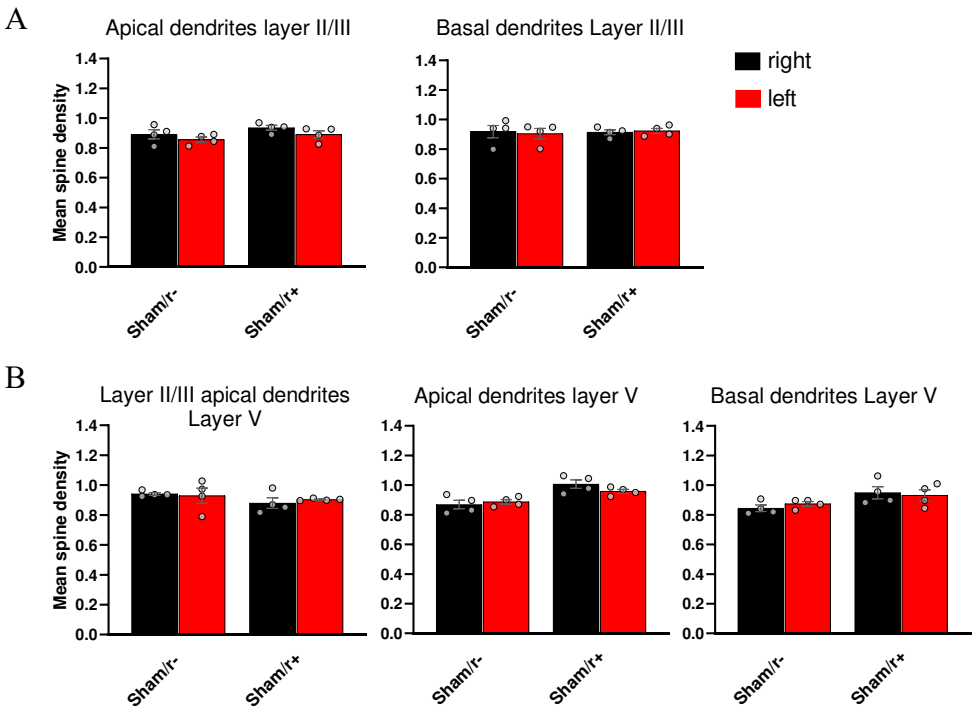

Figure 18. Dendritic spine density in Sham/r- and Sham/r+ groups in aged mice. A. The mean spine density in the distal apical (on the left) and basal (on the right) dendrites of layer II/III pyramidal neurons. ***B.*** The mean spine density in layer II/III apical (on the left), layer V apical (in the middle) and basal (on the right) dendrites of layer V M1 pyramidal neurons. Gray dots indicate individual values (n=4/group). Data are expressed as mean ± SEM. Analysis with one-way ANOVA followed by Tukey's Post hoc correction.

On the contrary, A-tDCS increased the mean spine density in layer II/III apical (One-way ANOVA followed by Tukey's post hoc correction. $F_{3,12} = 16.49$, *p=0.0003*) and basal dendrites ($F_{3,12} = 16.49$, *p=8.819*) (***Figure* 19A**), similarly to young mice group. On the contrary, the combination of tDCS and physical activity resulted in a larger number of spines in the unstimulated hemisphere in respect to the A-tDCS/r- unstimulated M1 (*p=0.04*) in apical dendrites, even if the left M1 maintained a lower spine density respect to the stimulated hemisphere (*p=0.04*) (***Figure 19A)***. When analyzing layer V, no effects of stimulation and/or walking was found (***Figure 19B***).

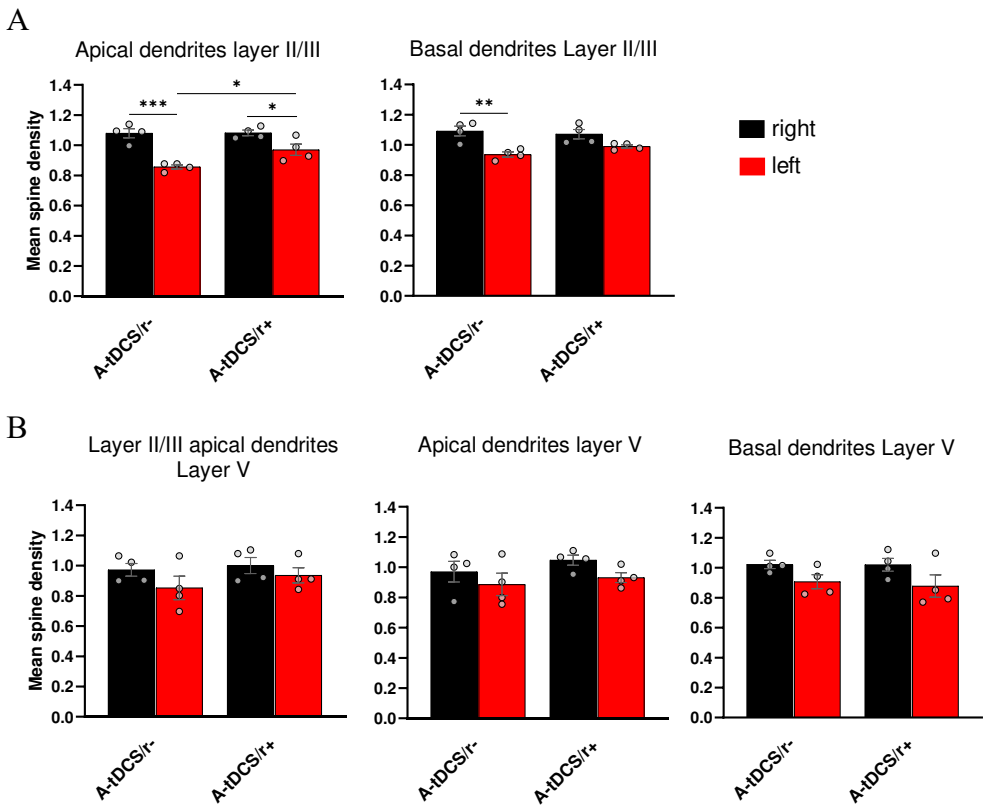

Figure 19. tDCS combined with a physiological motor cortex activation boost dendritic spine density in both hemispheres of layer II/III in aged mice. A. The mean spine density in the distal apical (on the left) and basal (on the right) dendrites of layer II/III pyramidal neurons. ***B.*** The mean spine density in layer II/III apical (on the left), layer V apical (in the middle) and basal (on the right) dendrites of layer V M1 pyramidal neurons. Gray dots indicate individual values (n=4/group). Data are expressed as mean ± SEM. Analysis with one-way ANOVA followed by Tukey's Post hoc correction. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Finally, investigation of PDS-95+ area in aging mice showed a similar trend to that of healthy young adults in both layer II/III and layer V (***Figure 20***), indicating that tDCS combined with activated motor cortex has similar effects in young and aging mice. In fact, PSD-95 signal was increased in the right M1 in layer II/III, with a significantly lower percentage only in the left hemisphere in the A-tDCS/r- condition ($p=0.01$, unpaired t test), while A/r+ showed similar levels of PSD-95 expression (***Figure 20A***). As for spine data, in layer V PDS-95+ area did not differ between conditions (***Figure 20B***).

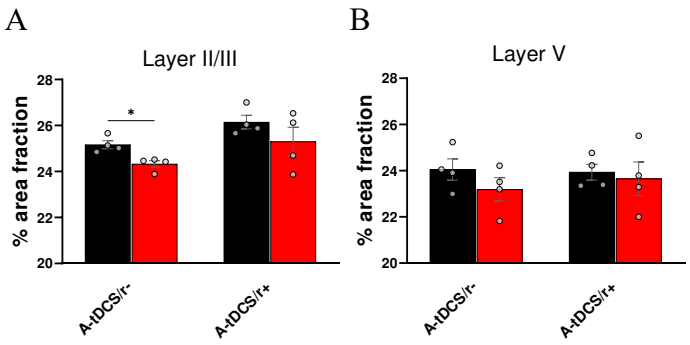

Figure 20. tDCS combined with a physiological motor cortex activation increases the percentage of PSD95+ area fraction in layer II/III in aged mice. ***A, B***. The percentage of area fraction of right and left layer II/III ***(A)*** and of layer V ***(B)*** M1 in A-tDCS/r- and A-tDCS/r+ conditions. Grey dots indicate individual values (n=4 per group). Data are expressed as mean ± SEM. Analysis with unpaired-t test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

5. DISCUSSION

The main finding of the present study is that the combination of M1 A-tDCS and physical activity positively affects cell activation, functional connectivity, and structural plasticity not only in the stimulated cortex but, more interestingly, in the non-stimulated M1.
This inter-hemispheric boosting effect does not occur when mice receive the stimulation alone, supporting the idea that tDCS effects strongly depend on the ongoing network activity (i.e. physiological activation of the motor cortices). Hence, this approach acts in a synergic manner, with addictive effects in respect to the mere summation of the two interventions.

Coupling tDCS and physical activity enhances motor cortex activation

The idea that combining tDCS and endogenous brain activation (e.g. motor activity) may have a synergistic addictive effect raised in recent human studies, in which physical exercise has been combined with tDCS in healthy volunteers (Wang et al., 2021). Nevertheless, the mechanisms responsible for this synergistic effect are not clear and animal studies on this topic are still missing.
For this reason, in this study we investigated the effects of the monolateral M1 A-tDCS, when the stimulation is applied alone or combined with a moderate physical activity (i.e. the rotarod at fixed speed).
Four experimental groups were considered: Sham/r-, which received sham stimulation in their homecage; Sham/r+, sham-treated mice performing rotarod; A-tDCS/r-, A-tDCS mice in their home cage; A-tDCS/r+, stimulated mice while walking on the rotarod.

In young adult mice (2-3 months), we tested cell activation by measuring cFos levels in layer II/III and in layer V of M1. We firstly examined the effects of walking by comparing Sham/r- with Sham/r+ mice. For this analysis we merged the left and right M1 hemispheres as walking implies both motor cortices activation (Artoni et al., 2017); as expected, our values did not differ between motor cortices. We found that physical activity resulted in an increased number of cFos+ cells both in layer II/III and in layer V, that is in line with previous findings indicating that motor activity induces cFos expression in M1 (Kleim et al., 1996). When analyzing the group receiving the monolateral M1 stimulation in their home cage (A-tDCS/r-), we observed a higher cell activation in the ipsi-lateral, stimulated hemisphere in respect to the contralateral M1 both in layer II/III and in layer V. Our result is consistent with human and animal literature evidence, in which monolateral M1 A-tDCS enhances neural activity in the stimulated hemisphere, as evidenced by MEPs or IEGs (Barbati et al., 2020; Cambiaghi et al., 2010; Nitsche & Paulus, 2000). Of note, in a recent study A-tDCS was shown to affect c-Fos mRNA levels in the stimulated sensorimotor cortex but not in the contra lateral one (Kim et al., 2017);

our findings resulted in an effective higher cFos protein expression in the right M1. The fourth group (A-tDCS/r+) received both A-tDCS and physical activity contemporarily. In both layers, we observed a greater cell activation in the stimulated M1 in comparison to the homologous M1 in A-tDCS/r-. Interestingly, while remaining a significant interhemispheric difference, in the A-tDCS/r+ group the non-stimulated M1also showed a higher activation with respect to the non-stimulated M1 in A-tDCS/r- in layer II/III and layer V. This outcome was not observed with the stimulation alone, thus, our results effectively indicate a synergistic effect of the combo, as observed in human studies. In humans, in fact, the coupling of these two interventions has been proved to improve motor cortical excitability and motor performance, when compared to tDCS or training alone, in a synergistic manner (F. Steinberg et al., 2019; Wang et al., 2021).

In addition, our outcome points out that the brain state is a key aspect for tDCS effects. The theory of the '*brain state-dependent brain stimulation*', proposed by Bergmann in 2018, suggests that neurons' behavior is affected by the ongoing activity of the local, or even global, network to which they belong. This determines the neurons response to the incoming sensory stimuli but also the tDCS effects (Bergmann, 2018). Of note, the ongoing network activity is defined by the afferent pathways and cortico-cortical projections (Draguhn & Buzsáki, 2004; Schroeder & Lakatos, 2009). Considering our results, the physiological afferent activation of the motor pathway, due to rotarod walking, has determined the enhancement of A-tDCS effects in A-tDCS/r+ mice, not only in the stimulated M1 but also in the contralateral M1. This suggests that the synergistic effect observed with the coupling depends on the physical activity action, which makes the motor network more prone to respond to tDCS, supporting the concept of the brain state-dependency of tDCS effects.

The indirect contralateral cell activation that we observed can be explained with a literature study in which tDCS application activates the expression of cFos both beneath the electrode as also in the interconnected regions (Cambiaghi et al., 2020) and it is well known that pyramidal neurons in layer II/III and layer V are responsible for the M1-M1 transcallosal projections in a homotopic organization (Chovsepian et al., 2017), making the right and left M1 functionally connected.

In line with our findings, in humans tDCS has been already observed to affect the excitability of the contralateral, non-stimulated motor cortex (Lang et al., 2004).

Of note, even though the effects of tDCS alone or the combo were found in both layers, layer V cell activation is lesser than that in layer II/III, probably because layer V is farer from the stimulation application point and the weak current is not able to largely affect the neuronal population.

Combining tDCS and physical activity functional M1-M1 interaction

These activation outcomes prompted us to explore whether the bilateral M1 enhancement may be associated to inter-hemispheric functional changes. Functional connectivity, in fact, has been proposed to be a key factor for the maintenance of motor functions (Lee, 2022). To this aim we analyzed the LFP Δcoherence (post-stimulation – baseline), as a measure of interhemispheric simultaneous activity (i.e. increased coherence meaning higher M1-M1 synchrony), in Sham/r+, A-tDCS/r- and A-tDCS/r+ conditions according to the following frequency bands: delta (0,5-3,5 Hz), low theta (4-8 Hz), theta (8,5-12 Hz), alfa (12,5-15 Hz) and beta (15,5-20 Hz). The Δcoherence did not reveal a difference between Sham/r+ and A-tDCS/r- in any frequency range. On the contrary, A/r+ mice showed increased coherence levels in the low-theta band (4-8 Hz), but not in the other frequencies. These results suggest that, when combining A-tDCS and physical activity, the motor cortex is more synchronized in the theta frequency band and the combo affects the functional M1-M1 connectivity.

Our findings are sustained by recent evidence indicating that tDCS is able to modulate entire brain networks and the functional connectivity in a non-invasive manner (Lewis et al., 2016; To et al., 2018). Additionally, the theta frequency band has already been linked to the motor-related activity in rodents. An increased theta activity has been associated, in fact, to the onset of locomotion and its power to the speed of the movement (Noga et al., 2017). Initially, theta rhythm was shown to be prominent in hippocampus (Kramis et al., 1975) and in the hypothalamic locomotor regions (Slawinska & Kasicki, 1995; Sławińska & Kasicki, 1998). However, it is accepted that theta rhythm extends to the other interconnected regions of the motor network, including the motor cortices (Bland & Oddie, 2001; Johnson et al., 2013; Noga et al., 2017). Thus, our results show that the combo of A-tDCS and physical activity leads to an enhancement of the functional M1-M1 connectivity, with a higher synchronization at the frequencies associated to locomotion.

This change in the oscillatory rhythmic activity may also indicate a functional experience-dependent remodeling of the neural circuit induced by the synergistic combo A-tDCS and physical activity.

tDCS combined with the physiological motor cortex activation boost dendritic spine density in both hemispheres

In mice, motor experience-dependent changes of the neural circuits have also been associated to synaptic plasticity (Cirillo et al., 2017; Rroji et al., 2015) and to dendritic spine dynamics, as the change in spine number (Clark et al., 2018; Ma et al., 2016; Tjia et al., 2017; Yang et al., 2009).

Dendritic spines represent the postsynaptic partner of most of the excitatory synaptic input (> 90%) and are highly modifiable (i.e. plastic) (Fortin et al., 2012; Yu & Zuo, 2011). Motor training has been observed to increase spine dynamics in the M1 (Clark et al., 2018; Ma et al., 2016; Xu et al., 2009; Yang et al., 2009), and A-tDCS has been shown to enhance dendritic spine density in the motor cortex too (Barbati et al., 2020). However, no data are currently available about the effects of combining A-tDCS with moderate physical activity on the structural plasticity. For this reason, we analyzed the apical and basal dendritic spine density in layer II/III and in layer V pyramidal neurons of both right and left M1 in young adult mice.

At first, we compared S/r- and S/r+ groups and we found that physical activity alone did not affect the mean spine density in both the considered layers. Considering the layer II/III, our result differs from the study of Ma et colleagues in which they found an increase spine density in M1 layer II/III apical dendrites after a single session of treadmill training at a constant moderate speed. However, their treadmill protocol (40 minutes with a 1-minute break every 5 minutes) is different from ours, consisting of two daily sessions of 10 minutes with three hours break in between for two consecutive days. Moreover, Ma et others compared the rate of spine formation over 24 hours before and after the treadmill training in young mice (Ma et al., 2016). On the contrary, we perfused the animals 90 minutes after the end of the last day of stimulation, so we did not follow the rate of formation and elimination of the spines over the 24 hours in our experimental protocol. These two reasons may explain why our outcomes are not coherent with literature evidence in layer II/III.

In addition, we used a moderate, constant rotarod speed for our experiments and a result like ours was obtained by Yang et colleagues in mice subjected to slowly constant speed rotarod. In fact, they observed the increase in spine density only with the accelerated rotarod in the apical layer V dendrites, even though no data on layer II/III spine dynamics was furnished (Yang et al., 2009).

The previous reported studies investigated the effects of physical activity on the structural plasticity in the apical but not in the basal dendrites, in fact, data on the structural basal dendritic plasticity are lacking in respect to apical dendrites (Tjia et al., 2017).

Considering the A/r- group, A-tDCS alone increased the Ipsi-lateral mean spine density in both layer II/III basal and apical dendrites, similarly to what already observed after 3 daily sessions of A-tDCS in awake mice (Barbati et al., 2020). On the contrary, in A/r+ mice, the combination between A-tDCS and physical activity resulted in a higher number of spines of the entire M1, left and right hemispheres, in both apical and basal dendrites.

Altogether, these data demonstrate an indirect contra-lateral boosting effect of A-tDCS but only when it is coupled with the physiological activation of the motor pathway. This supports again the synergistic action of the combo on structural plasticity in this case and the importance of the activation

state of the network when tDCS is applied. In this context, evidence from Gellner et colleagues has already enlightened that A-tDCS applied over the sensorimotor cortex in combination with the contralateral electrical forepaw stimulation (eFS) rapidly increased the spine density in the apical dendrites of ipsi-lateral layer V pyramidal neurons in anesthetized mice. However, when tDCS or eFS was applied singularly, no changes in dendritic spine density were noted (Gellner et al., 2020). Our results are in accordance with this finding, even if we did not activate the afferent motor pathway with an external peripheral stimulation, instead we took advantage of the physiological activation of the same afferent pathway. Another difference is that our mice were awake and not anesthetized. Nevertheless, both the studies support that the afferent input can effectively modulate tDCS effects.

The dendritic spine density in layer V did not change neither in the Sham/r+ nor in the A-tDCS/r- group, analogously to the previous experiment of Gellner et colleagues on anesthetized mice. We did not find a difference in A-tDCS/r+ anyway. It may be due to many reasons, both technical and physiological. Firstly, layer V is a deep layer, and its neurons are located farer from the active electrode than those in layer II/III. Thus, the weak current of the tDCS could not reach layer V in an efficient way to induce an effect on the structural spine plasticity.
More likely, our approach has an effect on cell activation of layer V, as observed in cFos+ cell count, but it is not sufficient to induce dendritic spine formation. In addition, it is known that layer II/III and layer V pyramidal neurons have different properties: in young adult the spine dynamic is higher in layer II/III pyramidal neurons with respect to the neurons in layer V (Tjia et al., 2017), thus, layer II/III is more plastic (Rioult-Pedotti et al., 1998). This could also explain our results, as it indicates that layer V neurons are less plastic-inducible via our protocol of stimulation. Of note, the motor experience-dependent dendritic spine remodeling and spine dynamics of layer II/III pyramidal neurons is less known than the one in layer V (Tjia et al., 2017). Here, we demonstrated that apical and basal dendrites of layer II/III in the M1 are affected by the combination of A-tDCS and moderate physical activity more than layer V pyramidal neurons.

Summarizing, the dendritic spine density of layer II/III is strongly influenced by the coupling between A-tDCS and motor activity. In dendrites, the post-synaptic scaffolding protein PSD95 play a key role in the stabilization of excitatory synapses, in the synaptic functionality and in maintaining the organization of the post-synaptic density (Cingolani & Goda, 2008; Ehrlich et al., 2007). Matsuzaki and others found that glutamate uncaging induced increases in both synaptic currents and spine sizes, mediated by postsynaptic neuronal processes, providing the first evidence of structure-function relationship in individual spines (Matsuzaki et al., 2004). Thus, to measure to what extent the

increased spine density is related to a synaptic potentiation when A-tDCS is applied, we analyzed the M1 percentage of PSD95+ fraction area in A-tDCS/r- and A-tDCS/r+ conditions in layer II/III and layer V. In layer II/III, PSD95 signal was increased in the right hemisphere of both conditions, with a significant lower percentage only in the left M1 of the A-tDCS/r- condition which was not observed in the A-tDCS/r+ group. In layer V, PSD95+ area did not differ between conditions. Our data confirm our previous findings of the mean spine density, indirectly indicating a higher synaptic function and an enhanced synapse stabilization mechanism.

The effects of the coupling in physiological aging

Physiological aging implies an impairment in balance, motor coordination and gait (Broom et al., 2021; Takahashi et al., 2016). In rodents, this age-related motor function decline has been associated to alterations in motor cortical excitability. One presumable underlying mechanism is an altered synaptic plasticity, related to the reduction of spine density in aged brains (van der Zee, 2015). However, in a recent study by Davidson et colleagues, the researchers found an increased spine density in the aged group, along with an elevated spine turnover and short-term stabilization of spines (Davidson et al., 2020). They proposed that this short-term spine stabilization could be a compensatory mechanism, as if the aged mice cortex may be in a perpetual state of instability and attempted compensation. Hence, the structural plasticity in aged mice remains controversial. Additionally, in humans an age-dependent decline of tDCS-induced plasticity effects was observed, but animal studies are still lacking.

Here, we applied our combined protocol to elderly to verify if the synergistic effects still occur when the physiological aging alters the state of the network. To this aim we tested motor cortex activation and dendritic spine density to aged mice (15 ± 2 months). Firstly, as for young adult mice, we observed the cell activation through cFos analysis in both layer II/III and layer V of the M1. The outcomes in layer II/III are similar to those obtained in young adults, indicating that our approach can likely affect cell activation in layer II/III at both ages.

Besides, the mean spine density in apical and basal dendrites of layer II/III resembles the one in younger mice, even if originating from a lower spine density. These findings indicate that in our mice the physiological aging causes a reduction of spine density in the M1, as suggested by Van der Zee et other (van der Zee, 2015), and that the motor network is altered in physiological aging, as already observed in literature (Albertson et al., 2022). Nevertheless, the effects of tDCS in layer II/III are not affected by the altered state of the circuit and the combination between A-tDCS and physical activity maintains the synergic effects in cell activation and structural plasticity.

Considering cell activation in layer V, together with no effects of walking, we observed A-tDCS effects in the A-tDCS/r+ condition, in which the right hemisphere showed a significant higher activation with respect to the left hemisphere. No differences were observed in the A-tDCS/r- group. Even if motor activity does not significantly change cell activation, a slight increase in respect to Sham/r- condition is present. It is possible that the highly variable data distribution is not allowing to statistically infer the action of the physical activity on the cell activation in elderly group. A non-significant difference between the right and the left M1 in the A-tDCS/r- condition was also observed, contrarily to young adult mice in which, even if lesser than in layer II/III, a difference was present. This points out that tDCS plasticity effects are age-dependent, as suggested in recent human studies (Ghasemian-Shirvan et al., 2020). The combination of tDCS and physical activity maintains an effect, as in A-tDCS/r+ condition the cell activation is increased in the right hemisphere mainly, with a mild, non-significant increase in the left M1 when compared to the left M1 in A-tDCS/r- condition. Dendritic spine density in layer V reflects the achievement in young adult mice, as no differences were observed. The PSD95+ area fraction supports the results of the dendritic spine density for both the A-tDCS/r- and A-tDCS/r+ groups in layer II/III and in layer V. All our data indicate that tDCS-induced structural plasticity effects are effectively declined in elderly, as observed in human studies (Ghasemian-Shirvan et al., 2020) or, better, they are reduced in layer V pyramidal neurons. So that, layer V apparently is more affected by the physiological aging in comparison to layer II/III, at least in terms of tDCS-plasticity effects, as reduced cell activation and dendritic spine density was observed in this deep layer. Moreover, the synergy of the combo affects layer II/III pyramidal neurons, as in younger mice, but its effect is reduced in layer V, indicating that the network alteration leads to a lower efficiency in responding to the coupling in layer V but not in layer II/III. However, the coupling has an inter-hemispheric effect in layer II/III which is not present in A-tDCS/r- group, this could explain why in humans the combination of the approaches showed an amelioration of the motor performance in elderly (Parikh & Cole, 2014).

Final considerations

Altogether, our outcomes strongly indicate that the state of the motor network is a key aspect for the tDCS effects, in fact, when tDCS is applied in combination with the walking, mice displayed a higher cell activation and structural plasticity mainly in layer II/III, not only in the right hemisphere but also in the contralateral unstimulated hemisphere. Contrarily, these intra- and inter-hemispheric enhanced effects were not observed whit tDCS alone, which causes a lower effect only in the ipsi-lateral M1. Thus, motor activity physiologically activates the motor network which, in turn, determine the tDCS-

induced plasticity effects. This leads to the synergistic boosting effects of the coupling, as observed in human studies. Functional connectivity is also affected by the coupling, with an enhanced M1-M1 synchronization in the theta rhythm, associated to locomotion, that is not present with A-tDCS alone. The plasticity results obtained for the layer II/III in young adult mice are resembled in the aged group, indicating that the synergistic effects of the combo are still efficacious even if the motor network plasticity is reduced.

Limitations of the present study

- Considering our stimulation protocol, we used a current density and an electrode montage that falls within the safety range suggested by anodal stimulation in rats (Liebetanz et al., 2006), and adopted for animal models (Cambiaghi et al., 2010; Barbati et al., 2020). We applied a stimulation of 240μA for 10 minutes, twice a day for two consecutive days and we didn't find any histological lesions in the cortical region underneath the epicranial electrode, as assessed by immunofluorescent and Nissl staining. Thus, the stimulation parameters used can be considered to promote physiological activity without inducing cellular damage. Nevertheless, rodent epicranial stimulation is regarded as an 'hypersensitive model' (Paciello et al., 2018) as the current intensity is higher than the one used in humans.

  Another difference in respect to the human studies is the montage for the delivering of the stimulation. Here, we used two distant electrodes to avoid current shunting over the mice head (Miranda et al., 2006), whereas a bilateral montage is usually adopted in human experiments (Nitsche & Paulus, 2000). These differences should be considered for clinical comparison or translational approaches.
- In human studies, the combination of A-tDCS and physical activity has been proved to enhance motor performance in healthy young volunteers (Wang et al., 2021) and in elderly (Hummel et al., 2010; Parikh & Cole, 2014). In the present research study, we analyzed the effects of the coupling on motor cortex activation and plasticity in both young and aged mice, but we did not evaluate the eventual motor performance improvement associated.

  Another issue is the effect of the combination on motor skill learning, for example in a fine reaching task as the pasta matrix. As observed in literature, structural plasticity of layer V apical dendrites have been strongly associated to improve in motor skill learning (Clark et al., 2018; Xu et al., 2009; Yang et al., 2009), as well as layer II/III (Peters et al., 2014; Rioult-Pedotti et al., 2000; Tjia et al., 2017). It may be interesting to investigate the possible improvement in learning due to the synergistic effects of the coupling.

- Another open question is if the newly formed spines that we observed in layer II/III will be stabilized or not. We perfused the animals 90 minutes after the end of the last stimulation, so we do not know if the increased spine density will be maintained. Gellner et colleagues demonstrated that A-tDCS in combination with the eFS induced an enhanced spine density that lasted for 24 hours after the stimulation. Specifically, both an increase of the original spine survival and the formation of new spines, morphologically characterized by larger head sizes, were observed (Gellner et al., 2020). Thus, we could suppose that they can last at least for 24 hours, but our approach is completely different, and our mice are awake and not under anesthesia.
- The functional connectivity in elderly is missing. Currently, we are implementing the study with the LFP coherence data in aged mice, as it was shown in literature that the homotopic inter-hemispheric functional connectivity is altered with the physiological aging (Albertson et al., 2022).

Future perspectives

In the present PhD thesis, the synergistic effects of coupling A-tDCS and physical activity have been verified in physiological conditions. However, since our findings proved an inter-hemispheric boosting effect, it could be noteworthy to apply this approach to pathological conditions, especially for rehabilitation purposes after stroke. In fact, this inter-hemispheric effect may allow to act on the ischemic hemisphere through the stimulation of the healthy hemisphere, possibly enabling to reduce the inter-hemispheric imbalance that is typical of the chronic stroke.

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