KU Leuven
Biomedical Sciences Group
Faculty of Kinesiology and Rehabilitation Sciences
Department of Kinesiology

IMPROVING NEUROPLASTICITY AND MOTOR LEARNING BY BRAIN STIMULATION TECHNIQUES

Orjon Rroji

Promoters:
Prof. Dr. Nicole Wenderoth
Prof. Dr. Bart Nuttin
PhD Kris van Kuyck

Dissertation presented in partial fulfillment of
the requirements for the degree of Doctor in
Biomedical Sciences

February 2016

Jury members:

Promoters: Prof. Dr. N. Wenderoth

Prof. Dr. Bart Nuttin

PhD. K. van Kuyck

Chair   Prof. Dr. J. Lefevre

Jury members: Prof. Dr. D. Balschun

Prof. Dr. R. Krampe

Prof. Dr. H. Feys

External jury member: Prof. Dr. R. Meeusen

Leuven, February 2016

Contents

Abbreviations

ANOVA	Analysis of variance
APB	Abductor pollicis brevis
BDNF	Brain-derived neurotrophic factor
EMG	Electromyography
LTD	Long-term depression
LTP	Long-term potentiation
M1	Primary motor cortex
MEP	Motor evoked potentials
NMDA	N-methyl-D-aspartate
RT	Retention test
tDCS	transcranial Direct Current Stimulation
TMS	Transcranial Magnetic Stimulation

English summary

Neuroplasticity refers to the ability of the brain to change as a result of one's experience, indicating that the brain is plastic and malleable. Synaptic plasticity is the ability of synapses to strengthen or weaken over time, in response to increases or decreases of activity. In the clinical context it determines how patients with a brain injury can recover, e. g. after stroke, in order to regain independence and to perform daily life activities (e.g. dressing, eating, self-care and personal hygiene). Previous studies have demonstrated that plasticity can be enhanced by different mechanisms.

In this PhD project we tested the effectiveness of non-invasive brain stimulation techniques to influence neuro-plasticity.

First, we tested reward related interventions which previously have been proved to boost neuroplasticity. For example monetary reward has been shown to improve the acquisition and particularly long-term retention of a newly acquired motor skill in humans. The physiological substrate mediating this effect is most likely dopamine (DA), a neuromodulator influencing cognitive, emotional, motivational and motor processes.

Secondly, we tested the effect of transcranial Direct Current Stimulation (tDCS), known to promote neuroplasticity, in healthy young volunteers. Previous research suggests that anodal tDCS over the primary motor cortex (M1) modulates NMDA receptor dependent processes that mediate synaptic plasticity. We tested this proposal by applying anodal versus sham tDCS while the subjects practiced to flex their thumb as fast as possible. The repetitive practice of this task has been shown to result in performance improvements that reflect use-dependent plasticity resulting from NMDA receptor mediated, long-term potentiation (LTP)-like processes. While, tDCS has received much attention because it can be easily applied in a clinical context, its underlying mechanisms are not clear yet. In order to explore its mechanisms of action we decided to develop an animal model.

In the third experiment, we developed an animal model of stroke rehabilitation that better mimics tDCS applications in humans. Here we aimed to develop an animal model where the effect of anodal tDCS over ipsilesional M1 is tested while animals perform goal-directed limb training. Accordingly, rats were trained on the pasta matrix reaching task, which allows the manipulation of limb use in order to mimic human clinical phenomena. We induced photothrombotic stroke in the M1 contralateral to the preferred limb. The photothrombotic stroke animal model aims to induce ischemic damage within a cortical area through photo-activation of a light-sensitive dye previously injected in the blood system.

We concluded that behavioural markers of use-dependent plasticity are surprisingly insensitive to monetary reward or punishment which might result from the nature of the task. Our data suggest that anodal tDCS facilitates long-term memory formation reflecting use-dependent plasticity, supporting the idea that anodal tDCS facilitates synaptic plasticity mediated by an LTP-like mechanism. Our data also showed that the application of anodal tDCS during post-stroke training on a reaching and grasping task in rats is feasible. tDCS is beneficial to upper limb recovery, only when the animals performed the grasp training. The availability of an animal model that can be used to closely mimic recovery training in stroke patients opens new avenues for gaining more mechanistic understanding of the underlying principles. Our results suggest that tDCS is a promising adjuvant therapy to facilitate motor recovery following stroke.

Dutch summary

Neuroplasticiteit verwijst naar het vermogen van het brein om zich aan te passen als gevolg van ervaringen. Dit geeft aan dat het brein plastisch en vervormbaar is. Met synaptische plasticiteit wordt bedoeld dat synapsen versterken of verzwakken als gevolg van een stijging of daling van activiteit. In de klinische context bepaalt synaptische plasticiteit hoe patiënten met een hersenletsel kunnen herstellen, bv. na een beroerte, om terug onafhankelijk te kunnen functioneren en om dagelijkse activiteiten uit te voeren (bv. aankleden, eten, zelfzorg en persoonlijke hygiëne). Eerdere studies hebben aangetoond dat plasticiteit kan worden verbeterd door verscheidene mechanismen.

In dit doctoraatsproject testten we in hoeverre niet-invasieve hersenstimulatie neuroplasticiteit kan beinvloeden.

Ten eerste testten we of beloningsinterventies neuroplasticiteit kunnen verhogen. Het werd reeds aangetoond bij mensen dat beloningen in de vorm van geldbedragen, het leren en in het bijzonder het behoud van nieuwe motorische vaardigheden op lange termijn kan verbeteren. Het fysiologische substraat dat dit effect bemiddelt, is waarschijnlijk dopamine (DA); een neuromodulator die cognitieve, emotionele, motivationele en motorische processen beïnvloedt.

Ten tweede testten we het effect van transcraniële gelijkstroomstimulatie (tDCS) bij gezonde jonge vrijwilligers. Eerder onderzoek suggereert dat anodale tDCS over de primaire motorische cortex (M1) synaptische plasticiteit kan bemiddelen door modulatie van NMDA receptor afhankelijke processen. We pasten anodale versus sham tDCS toe terwijl de proefpersonen herhaaldelijk flexiebewegingen van de duim uitvoerden. Het repetitieve oefenen van deze taak leidt tot prestatieverbeteringen en gebruiksafhankelijke plasticiteit vloeit vervolgens voort uit NMDA-receptor geïnitieerde langdurige potentiatie (LTP)-achtige processen. Ondanks de vele aandacht die tDCS verkreeg door de eenvoudige toepassing in een klinische context, zijn de onderliggende mechanismen nog niet duidelijk. Om de werkingsmechanismen te bestuderen, werd besloten een diermodel te ontwikkelen.

In het derde experiment onderzochten we beroerte in een diermodel. We trachtten het effect van anodale tDCS over M1 aan de zijde van de laesie te testen, terwijl ratten doelgerichte reiktaken uitvoerden met hun ledematen dmv een pasta matrix. Deze aanpak laat toe om het gebruik van de ledematen te manipuleren en om menselijke klinische verschijnselen na te bootsen. We induceerden een fototrombotische beroerte in M1 contralateraal van de gewenste ledematen. Deze

fototrombotische beroerte in het diermodel beoogt ischemische schade in een corticaal gebied te induceren via de foto-activatie van een in de bloedbaan ingespoten lichtgevoelige kleurstof.

We concludeerden dat gedragsmatige markers van gebruiksafhankelijke plasticiteit verrassend ongevoelig zijn voor monetaire beloning of straf, welke mogelijks kunnen voortvloeien uit de aard van de taak. Onze gegevens suggereren dat anodale tDCS langdurige geheugenvorming vergemakkelijkt wat gebruiksafhankelijke plasticiteit reflecteert. Dit ondersteunt het idee dat anodale tDCS synaptische plasticiteit vergemakkelijkt dmv een LTP-mechanisme. Onze gegevens toonden ook aan dat bij post-stroke ratten de toepassing van anodale tDCS tijdens het trainen van een reiktaak haalbaar is. Dit blijkt daarenboven gunstig te zijn voor het herstel van de bovenste ledematen, maar enkel wanneer de dieren de reiktaak trainen. De beschikbaarheid van een diermodel dat kan worden gebruikt om revalidatie training bij mensen nauwkeurig na te bootsen, opent nieuwe mogelijkheden voor het verkrijgen van een beter inzicht in het mechanisme en de onderliggende principes. Onze resultaten suggereren dat tDCS een veelbelovende aanvullende therapie is die motorisch herstel na een beroerte kan vergemakkelijken.

Chapter one

General introduction

Introduction

The consequences of stroke are often devastating, with the majority of survivors suffering from motor deficits. Stroke is the leading cause of long-term motor disability in adults worldwide (Clarke et al., 1999; Feigin et al., 2009). The recovery of motor function post stroke is often limited highlighting the need for more effective interventions (Kolominsky-Rabas et al., 2001). Over the past decade an increasing number of studies has sought to identify adjuvant therapies to boost neuroplasticity during recovery. In this project we investigated whether neuroplasticity can be enhanced by non-invasive interventions with the aim of facilitating motor recovery after stroke.

In this introductory chapter we will first provide an overview of neuroplasticity in the context of motor learning. We will then describe how markers of neuroplasticity can be measured non-invasively in humans. Thereafter we will outline the techniques used in the experimental chapters to modulate neuroplasticity, and how these might be useful in the case of stroke. Finally we will present a brief overview of subsequent chapters and the aims of the thesis.

Neuroplasticity in the healthy brain

Neuroplasticity is the ability of the brain to change in response to experience, to the environment, and to physiological modifications such as brain damage (Pascual-Leone et al., 2005; Sharma & Cohen, 2012). Although it was previously thought that the brain is a physiologically static organ, it is now understood that plasticity is a normal ongoing process throughout the life span (Pascual-Leone et al., 2005). Long-term potentiation (LTP) reflects an activity dependent increase in synaptic strength and is one important mechanism mediating neuroplasticity (Bliss & Collingridge, 1993).

It has been shown that LTP is dependent on the glutamatergic N-methyl-D-aspartate (NMDA) receptor (Coan & Collingridge, 1987). Glutamate activates NMDA receptors allowing calcium to enter the cell which acts on calmodulin-dependent protein kinases leading to the upregulation of AMPA receptors (Miyamoto, 2006). LTP is additionally modulated by the GABAergic system and Castro-Alamancos & Connors, (1996) showed that a reduction in GABA inhibition facilitates LTP-like activity in motor cortex.

LTP-like mechanisms can be activated in humans by repeated practice of motor actions that induce neural changes known as use-dependent plasticity. For example, use-dependent plasticity is clearly evident after several minutes of brisk thumb movements (Classen et al., 1998). Many studies have shown plasticity in motor cortical areas related to task learning (Dayan & Cohen, 2011; Kleim &

Jones, 2008). Animal studies in rodents have demonstrated that one day of prehension training results in changes in gene expression in the primary motor cortex in rats (Kleim et al., 1996), and three days of training are sufficient to induce an increase of evoked field potentials in the primary motor cortex (M1) of the trained limb (Rioult-Pedotti et al., 1998). Studies in humans have also shown that several days of training on a specific task induce cortical plasticity (Draganski et al., 2004; Scholz et al., 2009).

Motor training can lead to motor learning in both the healthy and damaged brain, inducing central nervous system plasticity (Dayan & Cohen, 2011; Zeiler & Krakauer, 2012). Plasticity can occur at all levels of the nervous system and which structure undergoes the largest plastic changes depends mainly on the practiced task. However, there is growing consensus that M1 – the area central for executing motor commands - is strongly effected by all types of motor training.

In the human motor cortex, motor training leads to encoding of the practiced movement (Classen et al., 1998). Encoding is a process associated with practice that results in the formation of motor memory (Bütefisch et al., 2004), and is thought to occur during the acquisition (practice) phase. Encoding is important as the learner processes information related to the task connecting the goal and movement outcome (Robertson, 2009). Following encoding, motor skills undergo consolidation, allowing new skills to be retained over time (Romano et al., 2010). Retention of the motor skill is usually assessed under the same conditions as during the acquisition phase (Kantak & Winstein, 2012). Several factors influence the long-term retention of skill learning. Reward during practice has been shown to improve long-term retention of a sequential motor skill (see below). Also, non-invasive brain stimulation techniques such as anodal transcranial Direct Current Stimulation (tDCS) can facilitate long-term memory formation in healthy subjects (see below).

Neuroplasticity during motor recovery after stroke

Motor learning is not only an important mechanism for the development of the healthy brain but it has been argued that the same processes are also essential for neurorehabilitation after a brain lesion, e.g. caused by stroke (Krakauer, 2006).

The damaged adult brain is able to reorganize and compensate for motor deficits in stroke patients (Calautti & Baro, 2003). While the cortical hemisphere contralateral to the lesion (i.e. contralesional) might play an important role initially, good recovery of hand function is typically characterized by increasing motor control exerted from the lesioned hemisphere (i.e. ipsilesional) (Bütefisch et al., 2005). Motor training is currently the most important intervention during the rehabilitation process, however, stroke recovery is a complex process that probably occurs through a combination of

factors. Even though repetition and training are fundamental for the brain and motor system to learn, there is not a clear recommendation concerning the duration and intensity of rehabilitation (Wahl et al., 2014). In humans and in animal models (rodents) there is a sensitive period post-stroke. Thus, the time course of motor recovery differs among animal and human studies. In animals the greatest recovery from impairment usually occurs in the first month (Biernaskie et al., 2004; Krakauer et al., 2012), while in humans it usually occurs in the first three months (Krakauer et al., 2012; Zeiler & Krakauer, 2012). Thus, rehabilitation during this critical period is fundamental for a significant recovery from impairment. Accordingly, Biernaskie et al., (2004) showed that the most significant gains in the recovery of the rat forelimb were achieved when rehabilitation started early (i.e. 5 days) compared to 30 days after stroke. Adkins & Jones, (2005) hypothesize that a longer lasting improvement would have been possible if the training started even earlier after lesion formation.

Probing motor cortex plasticity in humans using Transcranial Magnetic Stimulation (TMS)

TMS is a non-invasive, safe and painless way to stimulate human motor cortex (Barker et al., 1985). It is based on the principle of electromagnetic induction: a pulse of current is passed through the coil that is placed over a person's head, generating a rapidly changing magnetic field that penetrates the skull. The magnetic field induces current in the brain tissue under the coil, and depending on stimulation intensity, action potentials in stimulated neurons can be triggered (Kobayashi & Pascual-Leone, 2003). Different types of coils have been developed for TMS but a figure-of-eight coil shape is the most commonly used. When applied over the hand area of motor cortex, the coil is positioned tangentially to the scalp with the handle pointing backwards at an angle of 45° away from the mid-sagittal line. This position appears to be the most efficient for activating corticospinal pathways (Di Lazzaro et al., 2004; Rossini et al., 1994). TMS is commonly used to examine the integrity of the pathways connecting the brain and a muscle to evaluate damage in several diseases, such as stroke (Groppa et al., 2012). M1 can be focally stimulated by TMS, which generates motor evoked potentials (MEPs) in the target muscle (Stinear, 2010). The size of the MEP is a measure of corticomotor excitability. Use-dependent plasticity in humans causes reorganisation in primary motor cortex. In the case of intensive limb use, e.g. due to repetitive training, synapses become strengthened which is reflected by an increase of corticomotor excitability, that is, larger MEPs are observed after motor training than at baseline (Classen et al., 1998; Muellbacher et al., 2001; Rosenkranz et al., 2007). The increase in corticomotor excitability is most likely the result of training-induced activation of LTP-like mechanisms (Bütefisch et al., 2000; Stefan et al., 2006).

Modulators of neuroplasticity: **Reward and Punishment**

Reward and punishment are powerful modulators of human behaviour (Daw et al., 2002). Rewards can be classified into primary reward (e.g. food) and secondary reward (e.g. money). Primary rewards are innate and essential for daily life, while secondary rewards are not directly related to survival (Schultz et al., 2013). In decision making, actions are selected to optimize reward while minimizing cost or punishment (Frank et al., 2004; Pessiglione et al., 2006). This process relies on the dopaminergic midbrain and its projections to the prefrontal cortex (Pan et al., 2005; Schultz, 2002; Zaghloul et al., 2009). It has recently been shown that the influence of reward and punishment on behaviour also extends to motor learning tasks. Reward (i.e. earning money for good performance) has been found to improve learning and retention of a serial reaction time task (Wächter et al., 2009), and enhance the retention of a visuo-motor tracking task (Abe et al., 2011). On the other hand, punishment (i.e. losing money for poor performance) did not lead to a significant change in behaviour on either of these tasks. These results suggest that while reward can influence motor behaviour, punishment has little effect. However, it was recently shown that on an error-based motor adaptation task, reward did not influence learning gains but led to better retention, while punishment led to faster learning but did not influence retention (Galea et al., 2015).

Based on a large body of previous research most effects related to reward have been linked to the mesolimbic/mesocortical dopamine system: Reward processing is closely related to an increase in firing rate of midbrain dopaminergic neurons which project to cortex and particularly to primary motor cortex (M1) (Descarries et al., 1987).

Most of the dopaminergic cells develop from a single embryological cell group originating at the mesencephalic-diencephalic junction projecting to various forebrain targets (Hynes & Rosenthal, 1999). Mesodiencephalic dopamine neurons form a neuronal group that includes substantia nigra pars compacta (SN), the ventral tegmental area (VTA), and the retro-rubral field. The DA system includes the mesolimbic and mesocortical pathway which arise from VTA and these DA systems are involved in emotion-related behavior including motivation and reward (Mogenson et al., 1980; Phillips et al., 2008). In monkeys it was shown that M1 receives dense connections from dopaminergic neurons and that the number of D1 receptors (causing excitatory effects at the post-synaptic membrane) is roughly 10 times larger than the number of D2 receptors (causing inhibition at the post-synaptic membrane) (Gu, 2002). It has been proposed that when dopamine binds to D1 receptors, NMDA-receptor mediated responses to glutamatergic inputs are increased (Gu, 2002). Moreover, in vivo work has shown that dopaminergic projections from VTA to M1 are a prerequisite

for successful motor learning in rats (Molina-Luna et al., 2009). More specifically, selective destruction of VTA neurons that project to M1 of rats affected motor learning but not motor execution of over-trained movements (Hosp et al., 2011). Data showed that short-term improvement of performance within one session was not affected by the VTA lesion but long-term improvements were clearly diminished suggesting that DA is required for longer-lasting storage mechanisms. Compared to a control group, dopamine depletion of M1 reduced learning gains, however, this impairment could be resolved by substituting levodopa directly within M1 (Hosp et al., 2011; Molina-Luna et al., 2009). Thus, dopamine plays a key role in motor learning in animals.

In humans, it has been shown that VTA neurons respond to reward (Tobler et al., 2005) and that this activity influences decision making. However, much less is known about VTA-M1 interactions during motor learning except for first, indirect evidence based on behavioral studies showing that motor learning and retention are differentially influenced by reward and punishment (Abe et al., 2011; Galea et al., 2015).

Modulators of neuroplasticity: Neurophysiological effects of Transcranial Direct Current Stimulation (tDCS)

Transcranial Direct Current Stimulation (tDCS) is a non-invasive brain stimulation technique that delivers a low intensity constant current to the brain area of interest via electrodes on the scalp (Bindman et al., 1964; Landau et al., 1964; Nitsche & Paulus, 2000). It can be performed safely in animals and humans. In the 18th century with the invention of the electric battery, Galvani and Aldini conducted the first rudimentary studies in direct current stimulation. While Aldini and his collaborators used direct current to treat melancholia (Parent, 2004), its re-introduction as a medical therapeutic tool took place only in the 20th century. The pioneering work of Priori (Priori et al., 1998) followed by other researchers has demonstrated that tDCS can be applied to cortical areas (Paulus, 2003). tDCS differs from other plasticity inducing protocols (e.g. repetitive TMS) by not inducing neural action potentials because static fields in this range do not yield the rapid depolarization which is required to produce action potentials in neural membrane (Nitsche et al., 2008). Instead, tDCS modulates spontaneous neuronal network activity (Nitsche et al., 2008) by injecting low amplitude direct currents via one anodal and one cathodal scalp electrode (Fig 1). If the current flows from the active electrode to the reference electrode, it is named anodal. If the current flows from the reference electrode to the active electrode, it is named cathodal stimulation. Hereby, the anode is defined as the positively charged electrode, and the cathode is defined as the negatively charged

electrode. Anodal stimulation produces excitation (increases cortical excitability by depolarizing the membrane) and cathodal stimulation produces inhibition (decreases cortical excitability by hyperpolarizing the membrane) (Nitsche & Paulus, 2000, 2001).

 tDCS modulates spontaneous neuronal network activity rather than inducing neuronal firing (Nitsche et al., 2008). However, when anodal tDCS is applied to human motor cortex (M1) at rest it increases corticomotor excitability with after-effects outlasting the stimulation period by up to 2 hours (Nitsche & Paulus, 2001). These after-effects are synaptically driven, depend on the glutamatergic system and might be mediated by a long-term potentiation (LTP)-like mechanism (Nitsche et al., 2003). Previous research has shown that NMDA-glutamatergic receptors are involved in the facilitating effect induced by anodal tDCS and, in humans, anodal tDCS after-effects are abolished when NMDA receptors are blocked (Nitsche et al., 2003).

Anodal tDCS appears to modulate not only the glutamatergic synaptic transmission but also GABAergic activity. A positive correlation was observed between tDCS induced GABA decrease in M1 and degree of learning, i.e. showing a faster short term learning (Stagg et al., 2011). This finding matches the hypothesis that LTP-like plasticity within the neocortex is critically dependent on GABA modulation (Hess et al., 1996), i.e. synaptic strengthening is enhanced when GABAergic activity is suppressed.

Other neuromodulatory transmitters influencing cortical plasticity are acetylcholine (ACh), noradrenaline (NA), serotonin (5-HT), and histamine (Hist). Particularly ACh (Kuo et al., 2007) and 5-HT (Kuo et al., 2015) have been shown to enhance the effect of anodal tDCS on neuroplasticity in the motor cortex.

Another major player influencing cortical synaptic plasticity is brain derived neurotrophic factor (BDNF) (Lu, 2003). BDNF has been suggested to be a critical mediator of the anodal DCS effect based on in vitro experiments using M1 slices from adult mice carrying a forebrain specific deletion of the BDNF gene (postnatal excision of the floxed BDNF allele by Cre recombinase) (Fritsch et al, 2011). Slices derived from BDNF$^{flox/flox, cre}$ mice exhibited no synaptic potentiation after 15 min of tDCS exposure has been combined with a LTP inducing electrical stimulation protocol, whereas those from the Cre-negative BDNF$^{flox/flox}$ littermates displayed intact LTP. Based on these data they suggested that the facilitating effect of tDCS on synaptic plasticity is mediated by BDNF secretion in mouse M1 slices (Fritsch et al., 2011).

Activity-dependent BDNF secretion has been studied also in subjects with and without the BDNF Val66Met polymorphism, which is known to partially affect the activity-dependent secretion of BDNF (Chen et al., 2006). Previous research has associated the occurrence of a MET allele with a

reduction in practice-dependent learning (Fritsch et al, 2011) and also with a reduced response to anodal tDCS (Cheeran et al., 2008; Kleim et al., 2006).

Regarding practical aspects of applying anodal tDCS to human subjects, the polarity, duration, electrode position and intensity of tDCS can be controlled by the experimenter (Nitsche & Paulus, 2000). In studies on motor behaviour tDCS stimulation was primarily applied to M1. The current is typically applied via carbonized rubber electrodes covered with saline soaked sponges that vary in size from 25 to 35 cm^2. Both electrodes can be placed on the scalp (cephalic montage) or the reference electrode can be placed on another part of the body (extra cephalic montage). tDCS is usually applied for 5 – 30 min with an intensity varying between 1 – 2 mA. The stimulation can be given during one single training session (Hummel et al., 2010; Rroji et al., 2015; Tecchio et al., 2010) or during multiple training sessions (Reis et al., 2009; Saucedo Marquez et al., 2013). While tDCS is generally considered a safe technique there are some side effects such as tingling, moderate fatigue, light itching sensation, and less frequently headache, nausea and insomnia (Poreisz et al., 2007).

Figure 1 – Transcranial Direct Current Stimulation (tDCS) device. Figure taken from Newronika Company webpage.

tDCS in the healthy population

Many research groups have studied the effect of tDCS in the healthy population. The application of anodal tDCS placed over a specific region of the brain usually facilitates the learning of the task associated with that region (Reis et al., 2008). It has been demonstrated that tDCS increases corticomotor neural excitability (Nitsche & Paulus, 2001), and many studies have shown that anodal tDCS in combination with various types of motor tasks improves motor performance (Nitsche, et al., 2003; Antal et al., 2004; Reis et al., 2009; Saucedo Marquez et al., 2013; Tanaka et al., 2009).

Generally, anodal tDCS is effective in promoting long-term plastic changes associated with learning and memory formation (Wessel et al., 2015). However, it was shown that the effect of anodal tDCS applied to M1 is task specific (Bortoletto et al., 2015; Saucedo Marquez et al., 2013). Saucedo Marquez et al., (2013) tested the effect of anodal versus sham tDCS over M1 while the same individuals learned two different tasks, a sequential finger tapping task (SEQTAP) and a visual isometric pinch force task (FORCE). They showed that anodal tDCS over M1 enhanced learning gains for the SEQTAP task, while it improved long-term retention for the FORCE task. The authors speculate that long term memories of implicit sequence learning might strongly rely on the striatum (Doyon et al., 2009) whereas force control is believed to strongly rely on M1 (Sulzer et al., 2011). Accordingly task specific effects might arise because anodal tDCS has a stronger influence on neurons located directly underneath the electrode placed over M1, than on the neurons located further away from M1 (Saucedo Marquez et al., 2013).

The location of electrodes, the intensity and the duration, which all can vary, are also very important and influence the effect of tDCS (Nitsche & Paulus, 2000).

tDCS in stroke patients

The promising results obtained using non-invasive brain stimulation techniques in healthy subjects were extended to patients with neurological diseases, such as stroke. Previous research has demonstrated that motor learning might be an essential mechanism for neurorehabilitation (Krakauer, 2006), thus rendering anodal tDCS an interesting tool for modulating the response to rehabilitation training. In subacute (Hesse et al., 2011; Kim et al, 2009; Lee & Chun, 2014; Rossi et al., 2013) and chronic stroke patients (Bolognini et al., 2011; Hummel et al., 2005; Lindenberg et al., 2012; Fregni et al., 2005) different motor tasks (finger sequence learning, grasping tests) or functional tests (Box and Block test, Jebsen-Taylor Hand Function test, Wolf Motor Function Test, Fugl-Meyer Assessment) were performed in combination with tDCS. These different behavioural tests have shown that tDCS can improve motor function either when anodal stimulation is applied over ipsilesional M1, i.e. the anode was placed over M1 of the affected hemisphere and the cathode over the contralateral supraorbital area (Fregni et al., 2005; Hummel et al., 2005) or when anodal stimulation is applied to ipsilesional M1 and cathodal stimulation is applied to contralesional M1, (dual-tDCS) (Fregni et al., 2005; Lindenberg et al., 2010). The potential benefit of placing the anode over ipsilesional M1 and the cathode of contralesional M1 is twofold. First, anodal tDCS over the affected hemisphere might facilitate reorganisation and re-learning within motor circuits. Second, it has been hypothesized that stroke leads to an imbalance between hemispheres so that the more

affected hemisphere is suppressed by the less affected hemisphere (Murase et al., 2004). Applying anodal tDCS (i.e. facilitating stimulation) to the affected hemisphere, and cathodal tDCS (i.e. inhibitory stimulation) to the less-affected hemisphere might therefore counteract this hemispheric imbalance and aid recovery. Daily sessions or a single session of anodal tDCS or dual-tDCS (anode and cathode placed over each M1) have been shown to improve motor function in chronic stroke patients (Lefebvre et al., 2012; Lindenberg et al., 2010). tDCS may be able to improve motor function by upregulating the excitability of the lesioned motor cortex (Nowak et al., 2015). Although these are promising data, there is not yet clear evidence that bifocal stimulation (dual-tDCS) is more efficient than mono focal stimulation. The pilot study of Hesse et al., (2007) showed a promising result combining tDCS with robot-assisted arm training, but it was not possible to replicate the findings in a larger scale study (Hesse et al., 2011). The beneficial effects of tDCS were also shown in stroke patients with aphasia (Baker, 2010). In general, the published data are not consistent regarding the efficacy of tDCS in motor recovery (Feng et al., 2013).

tDCS in animals

Even though first results of applying tDCS in human stroke survivors are positive when analysed at the group level, there are large individual differences in the response to anodal tDCS. Currently, the field struggles to design tDCS applications that increase efficacy either at the group level or at the level of the individual because little is known about the underlying mechanism and important determinants. Since it is difficult to justify exploratory studies in patients suffering from stroke, there is an increasing need to develop adequate in vivo animal models which have proved useful for exploring the electrophysiological properties of tDCS. In vivo animal studies have been important for testing different combinations of stimulation parameters such as safety range, threshold for brain damage etc., which are important for brain stimulation research in general. Many experiments have tested the physiological effects of tDCS in healthy animals, for example, by measuring cerebral blood flow (CBF) while applying current intensities of 25, 50 and 100 µA. CBF was measured prior to and after tDCS for 30 min using laser Doppler flowmetry. At higher intensities anodal tDCS increased CBF up to 30 min. In contrast cathodal tDCS at low intensities led to a decrease of CBF. However, both stimulations led to significant changes in CBF up to 30 min, and depending on the polarity applied it was possible to decrease (cathodal tDCS) or increase (anodal tDCS) CBF (Wachter et al., 2011). Other studies examined the effectiveness of tDCS by combining it with functional magnetic resonance imaging (Takano et al., 2011). 400- or 40-µA currents were applied for 10 min with the anode

mounted over frontal cortex. The 400-µA stimulation significantly increased the signal in the frontal cortex as well as in the nucleus accumbens, a connected area in the ventral striatum (Takano et al., 2011). The effect of tDCS has also been evaluated in animal models of stroke (Jiang et al., 2012; Kim et al., 2010; Notturno et al., 2014; Peruzzotti-Jametti et al., 2013; Yoon et al., 2012). Jiang et al., (2012) reported a potential trend for increased synaptic plasticity in a tDCS group compared to sham group when tDCS was applied for 7 days or longer. They reported an increase in dendritic spine density in the anodal tDCS group, suggesting that tDCS might play a role in promoting neural plasticity. Yoon et al., (2012) showed that 5 days of tDCS versus sham stimulation facilitated recovery of gross motor function as measured by the beam balance test and the motor behavioural index with rats that started receiving stimulation 1 week post stroke faring better than those that started receiving stimulation 1 day post stroke. Moreover, they showed that anodal tDCS starting 1 week post stroke promotes Gap-43 expression in intact cortex. Gap-43 has been shown to play an important role in axonal growth and the formation of new connections. Kim at al., (2010) found no significant effect of anodal tDCS on motor function when applied early after stroke, however they used an unusually large stimulation electrode for rats (1 cm diameter). They reported that anodal tDCS had no effect on infarct size, but white matter axons were better preserved in rats that received anodal tDCS. However, one crucial limitation of the above studies is that tDCS was always applied during anaesthesia which is -at least based on the results of human studies- much less effective than when tDCS is combined with rehabilitation training. Thus, the field is in need of a rat model that better mimics tDCS applications in stroke patients. Research done in rodents mostly used a montage similar to that reported by Liebetanz et al., (2006), fixing the electrodes directly onto the skull of the animal. The active electrode size is typically 3.5 mm^2, and the reference electrode, a carbonized rubber electrode, is placed on the chest and held by a small jacket. Current intensity and the duration of the stimulation are of utmost importance. Liebetanz et al., (2009) investigated the safety limits of tDCS in the rat brain. Histological analyses of their study demonstrated that for cathodal stimulation, no pathological brain lesions were observed for current densities between 142.9 and 287 A/m^2. No neurotrauma was visible if the current intensity was between 1 – 100 µA, with the earliest lesion signs starting at 500 µA.

One has to note though that there are very important methodological differences between humans and animal studies. The current density typically applied in animals (34.2 A/m^2) is 85 times higher compare to humans (0.4 A/m^2) (Brunoni et al., 2011) because almost all studies using rats as models have used a current intensity at the medium to upper safety limits determined by Liebetanz et al., (2009). Our study followed the same general approach but used lower intensities than most previous studies (chapter 4). We applied a constant current of 0.05 mA (corresponding to a current density of

15.9 A/m^2) which was delivered through the anodal electrode for 30 min duration in each training session. Stimulation at this intensity and duration does not cause discomfort, pain or damage to neural tissue (Liebetanz et al., 2009) while it was shown to effectively modulate brain activity (Jiang et al., 2012; Kim et al., 2010; Notturno et al., 2014; Yoon et al., 2012).

Even though the above studies showed that anodal tDCS might modulate some markers of plasticity after stroke, they are all limited because tDCS was applied during anaesthesia. The purpose of the present study was to develop an animal model of stroke rehabilitation that better mimics tDCS applications in humans using a bedside-to-bench approach. Most importantly, we aimed to develop an animal model where the effect of anodal tDCS over ipsilesional M1 is tested while animals perform goal-directed upper limb training. Therefore, rats were trained on the pasta matrix reaching task, a sensitive behavioural assay that allows the manipulation of limb use in order to mimic human clinical phenomenon (Kerr & Tennant, 2014).

Chapter overview

In the present doctoral thesis we investigated whether non-invasive interventions in combination with motor training can enhance neuroplasticity in the primary motor cortex (M1) area of healthy young volunteers and in an animal model of stroke. We set out to generate new knowledge with regard to three major questions:

First, little is known whether training under rewarded or punished conditions causes measurable physiological effects in M1. In Chapter 2 we tested this idea using a task that is believed to increased synaptic efficacy as a consequence of repetitive use.

Second, anodal tDCS has been shown to facilitate learning of motor tasks but most of these tasks probed skill learning. However, another important component of learning are changes occurring as a consequence of increasingly using of a limb (for example, due to repetitive practice). In chapter 3 we tested whether anodal tDCS over M1 would enhance this specific aspect of motor learning which is an essential component of many daily life training sessions in sports and rehabilitation.

Third, anodal tDCS has been shown to facilitate motor learning in healthy subjects and patients. However, very little is known about the underlying mechanism or important determinants which might influence the effectiveness of anodal tDCS. One reason for this gap in knowledge is that there are only few, in-vivo animal models available to investigate the effect of tDCS on synaptic plasticity.

In chapter 4 we develop a rat model for testing the influence of anodal tDCS on recovery after stroke.

An overview of each chapter is provided in table 1.

	Subjects	Motor task	Neuroplasticity Modulation	Applied area	Sessions
Chapter 2	33 (17 female; humans)	Thumb flexion	Reward and Punishment	-	1
Chapter 3	14 (7 female; humans)	Thumb flexion	tDCS /sham tDCS	M1	2
Chapter 4	28 (all male; rats)	Pasta matrix	tDCS /sham tDCS	Ipsilesional M1	10

Table 1: Overview of experimental chapters.

Chapter 2: **Reward modulates changes in corticomotor excitability induced by motor practice**

We first addressed if reward has a positive effect on use-dependent plasticity resulting from motor training, which is believed to serve as a model for studying LTP-like plasticity in humans. We wanted to investigate how monetary reward modulates motor behaviour and corticomotor excitability in healthy young volunteers. Single pulse TMS was used to measure corticomotor excitability. Thirty-three subjects practiced fast ballistic flexions of their non-dominant thumb. Repeated practice of a motor action can induce use-dependent plasticity, which is evident after several minutes of brisk thumb movements. Subjects were assigned either to the reward or punishment group and performed 10 blocks of thumb flexion movements of their non-dominant hand. Retention tests were performed after 30 min, after 24 hours and one week later. Subjects in both groups learned and retained the motor behavioural task equally well. Surprisingly, the punishment group showed an increase in cortical excitability from baseline to post training, while the reward group exhibited a clear decrease.

Chapter 3: **Anodal tDCS over the primary motor cortex facilitates long-term memory formation reflecting use-dependent plasticity**

Does anodal tDCS applied to M1 affect use-dependent plasticity in the form of increased neural and behavioural efficiency as a consequence of repeated practice? Here we test this proposal by applying anodal versus sham tDCS while subjects practiced to flex the thumb as fast as possible (ballistic movements). Repetitive practice of this task has been shown to result in performance improvements that reflect use-dependent plasticity resulting from NMDA receptor mediated, long-term potentiation (LTP)-like processes. Using a double-blind within-subject cross-over design, subjects participated either in an anodal or a sham tDCS session which were at least 3 months apart. Sham or anodal tDCS (1 mA) was applied for 20 min during motor practice and retention was tested after 30 min, after 24 hours and one week later. All subjects improved performance during each of the two sessions and learning gains were similar. Our main result was that long term retention performance (i.e. 1 week after practice) is significantly better when practice is performed with anodal tDCS than with sham tDCS. Our data strongly suggest that anodal tDCS facilitates long-term memory formation reflecting use-dependent plasticity. Our results support the notion that anodal tDCS facilitates synaptic plasticity mediated by an LTP-like mechanism, which is in accordance with previous research. To gain a more mechanistic insight we developed an animal model where we tested the effect of tDCS in a photothrombotic stroke model (chapter 4).

Chapter 4: **The effect of tDCS on motor recovery in an animal model of stroke**

The purpose of the study described in this chapter was to evaluate the effect of tDCS in an animal model for stroke rehabilitation that mimics tDCS applications in humans. tDCS can augment synaptic plasticity in vitro and in vivo with effects being larger when it is applied while synapses are active (for example due to motor training in in vivo models) which is thought to result in enhanced LTP-like plasticity. However, it is currently unclear whether tDCS early after stroke is truly beneficial for functional recovery in humans and which mechanisms might drive this effect. This gap in knowledge results partly from the lack of animal models that are translatable to clinical applications. We induced photothrombotic stroke in M1 after 10 days of grasping training. Starting from the 4th day post-infarct, animals were trained for 10 more days while anodal or sham tDCS (0.05 mA) was concurrently applied for 30 min. Rats with large bilateral lesions made very few grasps after surgery. After removing these rats from the analysis, the anodal tDCS group was found to recover grasping

function significantly better compared to the sham tDCS group. Our main finding is that applying tDCS to the ipsilesional cortex during 30 min of motor training in a rat stroke model is feasible. Moreover, combining motor training with anodal tDCS benefits recovery when compared to sham tDCS.

Chapter 5: **General Discussion**

In this chapter we will summarize the main findings of the studies that form this PhD thesis. These results are discussed within the context of studies previously performed in humans and animals. Finally, suggestions for future work are provided.

Outline of the experimental chapters

In this thesis we investigated whether neuroplasticity can be enhanced by non-invasive interventions with the aim of facilitating motor recovery after stroke. At the beginning of our project we used a simple motor task that has been shown to induce use-dependent plasticity in the motor system of healthy participants. We explored whether neuroplastic processes can be enhanced either via rewarding/punishing feedback, which is believed to modulate dopaminergic circuits (experiment 1), or by anodal transcranial Direct Current Stimulation (tDCS), which is thought to modulate the induction of Long Term Potentiation (LTP) at the synaptic level (experiment 2).

Our initial experiments in young, healthy participants revealed that anodal tDCS over the primary motor cortex had a much stronger effect on motor behaviour than rewarding/punishing feedback and we decided to focus on investigating the plasticity enhancing effect of tDCS in further detail. In a bedside-to-bench approach, we developed a rat model of recovery from stroke using a rehabilitation program similar to that used in humans, i.e. applying anodal tDCS while upper limb stroke rehabilitation training was performed. Male Sprague-Dawley rats were chosen to induce photothrombotic stroke and to test the effect of tDCS while they were performing/trained on the pasta matrix reaching task. In the next paragraph we will briefly describe the experimental tools and paradigms central to this thesis.

Behavioural motor tasks

Thumb flexion task (chapter 2 & 3): Performing repetitive brisk thumb movements in a certain direction for several minutes (30 min) is believed to strengthen existing neural connections and to facilitate the creation of new ones within M1. This phenomenon is known as use-dependent plasticity in response to training (Classen et al., 1998). Subjects had to perform a ballistic thumb flexion movement with their non-dominant hand. A Polhemus Fastrak accelerometer sensor was fixed on the nail of the thumb to measure 3D kinematics and provide online feedback. From the 3D kinematics, the absolute velocity was calculated by $V_i = ((X_i-X_{i-1})^2 + (Y_i-Y_{i-1})^2 + (Z_i-Z_{i-1})^2)^{1/2} / (t_i-t_{i-1})$ where X, Y and Z represent the displacement in the three dimensions, t is the time and i is the index of the current data point. For each movement the velocity profile was displayed on a computer display in front of the subject to provide performance feedback. Additionally, the maximum velocity was calculated and displayed for all trials, such that subjects saw how their performance changed across training.

Subjects were instructed to flex their non-dominant thumb as fast as possible, making an isolated movement, i.e. without activating other synergistic muscles. Movements were performed in blocks, each consisting of 20 movements (1 movement every 3 sec). Each block lasted 1 minute and in between blocks there was a break of 1 minute to prevent fatigue.

Visual information on the performance of the actual trial and the progression throughout the whole block was provided on a screen in front of the subjects (chapter 3). In chapter 2, subjects did not see the performance history, instead they were informed about their "reward history", i.e. how much money that had won/lost during training.

The thumb movement task serves as an example for training-induced, use-dependent plasticity. Even though it is a strong simplification of real-life tasks, it involves similar neural mechanism (i.e. increase in synaptic efficacy) as activated by repetitive training used in sports or rehabilitation training.

Pasta matrix reaching task (chapter 4): Rats were placed into a transparent plastic box and had to grasp pieces of pasta through a slot. This task requires animals to make independent skilled use of the limb. Thus, the task allows for quantitative (number of successful reaches/transports) assessment of behavioural performance and provides a sensitive measure of both motor impairment and improvement after stroke (Ballermann et al., 2001; Tennant et al., 2012). Repeated practice of motor actions induces neural changes, known as use-dependent plasticity, activating LTP-like mechanisms. Animals are forced to grasp with the dominant forelimb, and use-dependent plasticity

becomes evident after performing the same grasping movement. Due to training, the existing connections are strengthened and new ones should be created within M1.

tDCS in humans

The anode electrode (5 x 5 cm) was located over the hand area of the M1, which was localized with TMS. The cathode electrode (11 x 9 cm) was located on the ipsilateral shoulder (extracephalic placement). Anodal tDCS of 1 mA (corresponding to a current density of 0.4 A/m^2) was applied for 20 min over the hand area of the primary motor cortex. In the anodal tDCS condition the current was ramped up to 1 mA over 12 s and then applied at this intensity continuously. In the sham tDCS condition the same ramp up procedure was applied but the current was ramped down after 12 s (chapter 3).

tDCS in rats

A tube (outer diameter 3.86 mm) was attached onto the skull (1.5 mm anterior to bregma and 2.5 mm lateral from midline). The anode electrode (2 mm diameter) was inserted into the tube, and the cathode electrode (2.5 x 2.5 cm) was placed onto the ventral thorax using a jacket. Anodal tDCS with an intensity of 0.05 mA (corresponding to a current density of 15.9 A/m^2) was applied for 30 min over the primary motor cortex of the ipsilesional cortex. Anodal tDCS was applied by manually ramping up current intensity over 5 s. For sham tDCS, the electrode montage was the same but no current was delivered.

References

Abe, M., Schambra, H., Wassermann, E. M., Luckenbaugh, D., Schweighofer, N., & Cohen, L. G. (2011). Reward improves long-term retention of a motor memory through induction of offline memory gains. *Current Biology*, *21*, 557–562. http://doi.org/10.1016/j.cub.2011.02.030

Adkins, D. L., & Jones, T. a. (2005). D-Amphetamine enhances skilled reaching after ischemic cortical lesions in rats. *Neuroscience Letters*, *380*, 214–218. http://doi.org/10.1016/j.neulet.2005.01.036

Baker, J. M., Rorden, C., & Fridriksson, J. (2010). Using transcranial direct-current stimulation to treat stroke patients with aphasia. *Stroke*, *41*(6), 1229–1236. http://doi.org/10.1161/STROKEAHA.109.576785

Ballermann, M., Metz, G. a S., McKenna, J. E., Klassen, F., & Whishaw, I. Q. (2001). The pasta matrix reaching task: A simple test for measuring skilled reaching distance, direction, and dexterity in rats. *Journal of Neuroscience Methods*, *106*, 39–45. http://doi.org/10.1016/S0165-0270(01)00326-0

Barker, a T., Jalinous, R., & Freeston, I. L. (1985). Non-invasive magnetic stimulation of human motor cortex. *Lancet*, *1*(8437), 1106–1107. http://doi.org/10.1016/S0140-6736(85)92413-4

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(5), 1245–1254. http://doi.org/10.1523/JNEUROSCI.3834-03.2004

Bindman, L. J., Lippold, O. C., & Redfearn, J. W. (1964). the Action of Brief Polarizing Currents on the Cerebral Cortex of the Rat (1) During Current Flow and (2) in the Production of Long-Lasting After-Effects. *The Journal of Physiology*, *172*, 369–382. http://doi.org/10.1234/12345678

Bolognini, N., Vallar, G., Casati, C., Latif, L. a., El-Nazer, R., Williams, J., … Fregni, F. (2011). Neurophysiological and Behavioral Effects of tDCS Combined With Constraint-Induced Movement Therapy in Poststroke Patients. *Neurorehabilitation and Neural Repair*, *25*(9), 819–829. http://doi.org/10.1177/1545968311411056

Bortoletto, M., Concetta, M., Rodella, C., & Miniussi, C. (2015). The Interaction With Task-induced Activity is More Important Than Polarization : A tDCS Study. *Brain Stimulation*, *8*(2), 269–276. http://doi.org/10.1016/j.brs.2014.11.006

Brunoni, A. R., Fregni, F., & Pagano, R. L. (2011). Translational research in transcranial direct current

stimulation (tDCS): A systematic review of studies in animals. *Reviews in the Neurosciences*, *22*(4), 471–481. http://doi.org/10.1515/RNS.2011.042

Bütefisch, C. M., Davis, B. C., Wise, S. P., Sawaki, L., Kopylev, L., Classen, J., … Bu, C. M. (2000). Mechanisms of use-dependent plasticity in the human motor cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(7), 3661–5. http://doi.org/10.1073/pnas.050350297

Bütefisch, C. M., Khurana, V., Kopylev, L., & Cohen, L. G. (2004). Enhancing encoding of a motor memory in the primary motor cortex by cortical stimulation. *Journal of Neurophysiology*, *91*, 2110–2116. http://doi.org/10.1152/jn.01038.2003

Bütefisch, C. M., Kleiser, R., Körber, B., Müller, K., Wittsack, H.-J., Hömberg, V., & Seitz, R. J. (2005). Recruitment of contralesional motor cortex in stroke patients with recovery of hand function. *Neurology*, *64*(6), 1067–1069. http://doi.org/10.1212/01.WNL.0000154603.48446.36

Calautti, C., & Baron, J.-C. (2003). Functional neuroimaging studies of motor recovery after stroke in adults: a review. *Stroke; a Journal of Cerebral Circulation*, *34*(6), 1553–1566. http://doi.org/10.1161/01.STR.0000071761.36075.A6

Castro-Alamancos, M. A., & Connors, B. W. (1996). Short-term synaptic enhancement and long-term potentiation in neocortex. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(3), 1335–1339. http://doi.org/10.1073/pnas.93.3.1335

Cheeran, B., Talelli, P., Mori, F., Koch, G., Suppa, A., Edwards, M., … Rothwell, J. C. (2008). A common polymorphism in the brain-derived neurotrophic factor gene (BDNF) modulates human cortical plasticity and the response to rTMS. *The Journal of Physiology*, *586*(Pt 23), 5717–5725. http://doi.org/10.1113/jphysiol.2008.159905

Chen, Z.-Y., Jing, D., Bath, K. G., Ieraci, A., Khan, T., Siao, C.-J., … Lee, F. S. (2006). Genetic Variant BDNF (Val66Met) Polymorphism Alters Anxiety-Related Behavior. *Science*, *314*(5796), 140–143. http://doi.org/10.1126/science.1129663

Clarke, P. J., Black, S. E., Badley, E. M., Lawrence, J. M., & Williams, J. I. (1999). Handicap in stroke survivors. *Disability and Rehabilitation*, *21*(3), 116–123. http://doi.org/10.1080/096382899297855

Classen, J., Liepert, J., Wise, S. P., Hallett, M., & Cohen, L. G. (1998). Rapid plasticity of human cortical movement representation induced by practice. *Journal of Neurophysiology*, *79*, 1117–1123. http://doi.org/citeulike-article-id:2143137

Coan, E. J., & Collingridge, G. L. (1987). RECEPTOR COMPONENT IN RAT OF N-METHYL-D-ASPARTATE SYNAPTIC TRANSMISSION SLICES HIPPOCAMPAL in the hippocampus . Mg * + is a potent NMDA antagonist4 *" and recently we have reported that distributed throughout the mammalian brain where they have been impli, *22*(I), 1–8.

Daw, N. D., Kakade, S., & Dayan, P. (2002). Opponent interactions between serotonin and dopamine. *Neural Networks*, *15*(4-6), 603–616. http://doi.org/10.1016/S0893-6080(02)00052-7

Dayan, E., & Cohen, L. G. (2011). Neuroplasticity subserving motor skill learning. *Neuron*, *72*(3), 443–454. http://doi.org/10.1016/j.neuron.2011.10.008

Descarries, L., Lemay, B., Doucet, G., & Berger, B. (1987). Regional and laminar density of the dopamine innervation in adult rat cerebral cortex. *Neuroscience*, *21*(3), 807–824. http://doi.org/10.1016/0306-4522(87)90038-8

Di Lazzaro, V., Oliviero, a., Pilato, F., Saturno, E., Dileone, M., Mazzone, P., … Rothwell, J. C. (2004). The physiological basis of transcranial motor cortex stimulation in conscious humans. *Clinical Neurophysiology*, *115*(2), 255–266. http://doi.org/10.1016/j.clinph.2003.10.009

Doyon, J., Bellec, P., Amsel, R., Penhune, V., Monchi, O., Carrier, J., … Benali, H. (2009). Contributions of the basal ganglia and functionally related brain structures to motor learning. *Behavioural Brain Research*, *199*(1), 61–75. http://doi.org/10.1016/j.bbr.2008.11.012

Draganski, B., Gaser, C., Busch, V., Schuierer, G., Bogdahn, U., & May, A. (2004). Changes in grey matter induced by training Newly honed juggling skills show up as a transient feature on a brain-imaging scan . *Nature*, *427*, 311–312. http://doi.org/10.1038/427311a

Feigin, V. L., Lawes, C. M., Bennett, D. a., Barker-Collo, S. L., & Parag, V. (2009). Worldwide stroke incidence and early case fatality reported in 56 population-based studies: a systematic review. *The Lancet Neurology*, *8*(4), 355–369. http://doi.org/10.1016/S1474-4422(09)70025-0

Feng, W. W., Bowden, M. G., & Kautz, S. (2013). Review of transcranial direct current stimulation in poststroke recovery. *Topics in Stroke Rehabilitation*, *20*(1), 68–77. http://doi.org/10.1310/tsr2001-68

Frank, M. J., Seeberger, L. C., & O'reilly, R. C. (2004). By carrot or by stick: cognitive reinforcement learning in parkinsonism. *Science (New York, N.Y.)*, *306*(5703), 1940–1943. http://doi.org/10.1126/science.1102941

Fregni, F., Boggio, P. S., Mansur, C. G., Wagner, T., Ferreira, M. J. L., Lima, M. C., … Pascual-Leone, A. (2005). Transcranial direct current stimulation of the unaffected hemisphere in stroke patients.

Neuroreport, *16*(14), 1551–1555. http://doi.org/10.1097/01.wnr.0000177010.44602.5e

Fritsch, B., Reis, J., Martinowich, K., Schambra, H. M., Ji, Y., Cohen, L. G., & Lu, B. (2011). Direct current stimulation promotes BDNF-dependent synaptic plasticity: Potential implications for motor learning. *NIH Public Access*, *66*(2), 198–204. http://doi.org/10.1016/j.neuron.2010.03.035.Direct

Galea, J. M., Mallia, E., Rothwell, J., & Diedrichsen, J. (2015). The dissociable effects of punishment and reward on motor learning. *Nature Neuroscience*, 8–10. http://doi.org/10.1038/nn.3956

Groppa, S., Oliviero, a., Eisen, a., Quartarone, a., Cohen, L. G., Mall, V., … Siebner, H. R. (2012). A practical guide to diagnostic transcranial magnetic stimulation: Report of an IFCN committee. *Clinical Neurophysiology*, *123*(5), 858–882. http://doi.org/10.1016/j.clinph.2012.01.010

Gu, Q. (2002). Neuromodulatory transmitter systems in the cortex and their role in cortical plasticity. *Neuroscience*, *111*(4), 815–835. http://doi.org/10.1016/S0306-4522(02)00026-X

Hess, G., Aizenman, C. D., & Donoghue, J. P. (1996). Conditions for the induction of long-term potentiation in layer II/III horizontal connections of the rat motor cortex. *Journal of Neurophysiology*, *75*(5), 1765–1778.

Hesse, S., Werner, C., Schonhardt, E. M., Bardeleben, A., Jenrich, W., & Kirker, S. G. B. (2007). Combined transcranial direct current stimulation and robot-assisted arm training in subacute stroke patients: a pilot study. *Restorative Neurology and Neuroscience*, *25*, 9–15. http://doi.org/10.1177/1545968311413906

Hesse, S., Werner, C., Schonhardt, E. M., Bardeleben, A., Jenrich, W., & Kirker, S. G. B. (2011). Combined transcranial direct current stimulation and robot-assisted arm training in subacute stroke patients: An Exploratory, Randomized Multicenter Trial. *Restorative Neurology and Neuroscience*, *25*(1), 9–15. http://doi.org/10.1177/1545968311413906

Hosp, J. a, Pekanovic, A., Rioult-Pedotti, M. S., & Luft, A. R. (2011). Dopaminergic projections from midbrain to primary motor cortex mediate motor skill learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(7), 2481–2487. http://doi.org/10.1523/JNEUROSCI.5411-10.2011

Hummel, F. C., Heise, K., Celnik, P., Floel, A., Gerloff, C., & Cohen, L. G. (2010). Facilitating skilled right hand motor function in older subjects by anodal polarization over the left primary motor cortex. *Neurobiology of Aging*, *31*(12), 2160–2168. http://doi.org/10.1016/j.neurobiolaging.2008.12.008

Hummel, F., Celnik, P., Giraux, P., Floel, A., Wu, W. H., Gerloff, C., & Cohen, L. G. (2005). Effects of non-invasive cortical stimulation on skilled motor function in chronic stroke. *Brain*, *128*, 490–499. http://doi.org/10.1093/brain/awh369

Hynes, M., & Rosenthal, A. (1999). Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Current Opinion in Neurobiology*, *9*(1), 26–36. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10072377

Jiang, T., Xu, R. X., Zhang, a. W., Di, W., Xiao, Z. J., Miao, J. Y., … Fang, Y. N. (2012). Effects of transcranial direct current stimulation on hemichannel pannexin-1 and neural plasticity in rat model of cerebral infarction. *Neuroscience*, *226*, 421–426. http://doi.org/10.1016/j.neuroscience.2012.09.035

Kantak, S. S., & Winstein, C. J. (2012). Learning-performance distinction and memory processes for motor skills: A focused review and perspective. *Behavioural Brain Research*, *228*(1), 219–231. http://doi.org/10.1016/j.bbr.2011.11.028

Kerr, A. L., & Tennant, K. a. (2014). Compensatory Limb Use and Behavioral Assessment of Motor Skill Learning Following Sensorimotor Cortex Injury in a Mouse Model of Ischemic Stroke. *Journal of Visualized Experiments*, (July), 1–7. http://doi.org/10.3791/51602

Kim, D. Y., Ohn, S. H., Yang, E. J., Park, C.-I., & Jung, K. J. (2009). Enhancing motor performance by anodal transcranial direct current stimulation in subacute stroke patients. *American Journal of Physical Medicine & Rehabilitation / Association of Academic Physiatrists*, *88*, 829–836. http://doi.org/10.1097/PHM.0b013e3181b811e3

Kim, S. J., Kim, B. K., Ko, Y. J., Bang, M. S., Kim, M. H., & Han, T. R. (2010). Functional and histologic changes after repeated transcranial direct current stimulation in rat stroke model. *Journal of Korean Medical Science*, *25*, 1499–1505. http://doi.org/10.3346/jkms.2010.25.10.1499

Kleim, J. a, Chan, S., Pringle, E., Schallert, K., Procaccio, V., Jimenez, R., & Cramer, S. C. (2006). BDNF val66met polymorphism is associated with modified experience-dependent plasticity in human motor cortex. *Nature Neuroscience*, *9*(6), 735–737. http://doi.org/10.1038/nn1699

Kleim, J. a, & Jones, T. a. (2008). Principles of experience-dependent neural plasticity: implications for rehabilitation after brain damage. *Journal of Speech, Language, and Hearing Research : JSLHR*, *51*(February 2008), S225–S239. http://doi.org/10.1044/1092-4388(2008/018)

Kleim, J. a., Lussnig, E., Schwarz, E. R., Comery, T. a., & Greenough, W. T. (1996). Synaptogenesis and Fos expression in the motor cortex of the adult rat after motor skill learning. *J.Neurosci.*,

16(14), 4529–4535. Retrieved from PM:8699262

Kobayashi, M., & Pascual-Leone, A. (2003). Transcranial magnetic stimulation in neurology. *Lancet Neurology*, *2*, 145–156. http://doi.org/10.1016/S1474-4422(03)00321-1

Kolominsky-Rabas, P. L., Weber, M., Gefeller, O., Neundoerfer, B., & Heuschmann, P. U. (2001). Epidemiology of Ischemic Stroke Subtypes, 2735–2740.

Krakauer, J. W. (2006). Motor learning: its relevance to stroke recovery and neurorehabilitation. *Current Opinion in Neurology*, *19*(1), 84–90. http://doi.org/10.1097/01.wco.0000200544.29915.cc

Krakauer, J. W., Carmichael, S. T., Corbett, D., & Wittenberg, G. F. (2012). Getting Neurorehabilitation Right: What Can Be Learned From Animal Models? *Neurorehabilitation and Neural Repair*, *26*, 923–931. http://doi.org/10.1177/1545968312440745

Kuo, H., Paulus, W., Batsikadze, G., Jamil, A., Kuo, M., & Nitsche, M. A. (2015). Chronic Enhancement of Serotonin Facilitates Excitatory Transcranial Direct Current Stimulation-Induced Neuroplasticity. *Neuropsychopharmacology*, 1–8. http://doi.org/10.1038/npp.2015.270

Kuo, M.-F., Grosch, J., Fregni, F., Paulus, W., & Nitsche, M. a. (2007). Focusing effect of acetylcholine on neuroplasticity in the human motor cortex. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(52), 14442–7. http://doi.org/10.1523/JNEUROSCI.4104-07.2007

Landau, W. M., Bishop, G. H., & Clare, M. H. (1964). Analysis of the Form and Distribution of Evoked Cortical Potentials Under the Influence of Polarizing Currents. *Journal of Neurophysiology*, *27*(03), 788–813.

Lee, S. J., & Chun, M. H. (2014). Combination transcranial direct current stimulation and virtual reality therapy for upper extremity training in patients with subacute stroke. *Archives of Physical Medicine and Rehabilitation*, *95*(3), 431–438. http://doi.org/10.1016/j.apmr.2013.10.027

Lefebvre, S., Laloux, P., Peeters, a, Desfontaines, P., Jamart, J., & Vandermeeren, Y. (2012). Dual-tDCS Enhances Online Motor Skill Learning and Long-Term Retention in Chronic Stroke Patients. *Frontiers in Human Neuroscience*, *6*(January), 343. http://doi.org/10.3389/fnhum.2012.00343

Liebetanz, D., Koch, R., Mayenfels, S., König, F., Paulus, W., & Nitsche, M. a. (2009). Safety limits of cathodal transcranial direct current stimulation in rats. *Clinical Neurophysiology*, *120*(6), 1161–

1167. http://doi.org/10.1016/j.clinph.2009.01.022

Lindenberg, R., Renga, V., Zhu, L. L., Nair, D., & Schlaug, G. (2010). Bihemispheric brain stimulation facilitates motor recovery in chronic stroke patients. *Neurology*, *75*(24), 2176–2184. http://doi.org/10.1212/WNL.0b013e318202013a

Lindenberg, R., Zhu, L. L., & Schlaug, G. (2012). Combined Central and Peripheral Stimulation to Facilitate Motor Recovery After Stroke: The Effect of Number of Sessions on Outcome. *Neurorehabilitation and Neural Repair*, *26*(5), 479–483. http://doi.org/10.1177/1545968311427568

Lu, B. (2003). Pro-region of neurotrophins: Role in synaptic modulation. *Neuron*, *39*(5), 735–738. http://doi.org/10.1016/S0896-6273(03)00538-5

Miyamoto, E. (2006). Molecular mechanism of neuronal plasticity: induction and maintenance of long-term potentiation in the hippocampus. *Journal of Pharmacological Sciences*, *100*(5), 433–442. http://doi.org/10.1254/jphs.CPJ06007X

Mogenson, G. J., Jones, D. L., & Yim, C. Y. (1980). From motivation to action: functional interface between the limbic system and the motor system. *Progress in Neurobiology*, *14*(2-3), 69–97. http://doi.org/10.1016/0301-0082(80)90018-0

Molina-Luna, K., Pekanovic, A., Röhrich, S., Hertler, B., Schubring-Giese, M., Rioult-Pedotti, M. S., & Luft, A. R. (2009). Dopamine in motor cortex is necessary for skill learning and synaptic plasticity. *PLoS ONE*, *4*(9). http://doi.org/10.1371/journal.pone.0007082

Muellbacher, W., Ziemann, U., Boroojerdi, B., Cohen, L., & Hallett, M. (2001). Role of the human motor cortex in rapid motor learning. *Experimental Brain Research. Experimentelle Hirnforschung. Experimentation Cerebrale*, *136*(4), 431–438. http://doi.org/10.1007/s002210000614

Murase, N., Duque, J., Mazzocchio, R., & Cohen, L. G. (2004). Influence of Interhemispheric Interactions on Motor Function in Chronic Stroke. *Annals of Neurology*, *55*, 400–409. http://doi.org/10.1002/ana.10848

Nitsche, M. a, Liebetanz, D., Antal, A., Lang, N., Tergau, F., & Paulus, W. (2003). Modulation of cortical excitability by weak direct current stimulation--technical, safety and functional aspects. *Supplements to Clinical Neurophysiology*, *56*, 255–276. http://doi.org/14677403

Nitsche, M. a, & Paulus, W. (2000). Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. *The Journal of Physiology*, *527 Pt 3*, 633–639.

http://doi.org/10.1111/j.1469-7793.2000.t01-1-00633.x

Nitsche, M. a, & Paulus, W. (2001). Sustained excitability elevations induced by transcranial DC motor cortex stimulation in humans. *Neurology*, *57*(10), 1899–1901. http://doi.org/10.1212/WNL.57.10.1899

Nitsche, M. a, Schauenburg, A., Lang, N., Liebetanz, D., Exner, C., Paulus, W., & Tergau, F. (2003). Facilitation of implicit motor learning by weak transcranial direct current stimulation of the primary motor cortex in the human. *Journal of Cognitive Neuroscience*, *15*(1998), 619–626. http://doi.org/10.1162/089892903321662994

Nitsche, M. a., Cohen, L. G., Wassermann, E. M., Priori, A., Lang, N., Antal, A., … Pascual-Leone, A. (2008). Transcranial direct current stimulation: State of the art 2008. *Brain Stimulation*, *1*, 206–223. http://doi.org/10.1016/j.brs.2008.06.004

Notturno, F., Pace, M., Zappasodi, F., Cam, E., Bassetti, C. L., & Uncini, A. (2014). Neuroprotective effect of cathodal transcranial direct current stimulation in a rat stroke model. *Journal of the Neurological Sciences*, *342*(1-2), 146–151. http://doi.org/10.1016/j.jns.2014.05.017

Nowak, D. A., Grefkes, C., Ameli, M., Fink, G. R., Stimulation, B., & Stroke, F. (2015). Interhemispheric Competition After Stroke : Brain Stimulation to Enhance Recovery of Function of the Affected Hand, 641–656.

Pan, W.-X., Schmidt, R., Wickens, J. R., & Hyland, B. I. (2005). Dopamine cells respond to predicted events during classical conditioning: evidence for eligibility traces in the reward-learning network. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(26), 6235–6242. http://doi.org/10.1523/JNEUROSCI.1478-05.2005

Parent, A. (2004). Giovanni Aldini: from animal electricity to human brain stimulation. *The Canadian Journal of Neurological Sciences. Le Journal Canadien Des Sciences Neurologiques*, *31*, 576–584.

Pascual-Leone, A., Amedi, A., Fregni, F., & Merabet, L. B. (2005). The plastic human brain cortex. *Annual Review of Neuroscience*, *28*, 377–401. http://doi.org/10.1146/annurev.neuro.27.070203.144216

Paulus, W. (2003). Transcranial direct current stimulation (tDCS). http://doi.org/10.1016/j.neuropsychologia.2015.02.002

Peruzzotti-Jametti, L., Cambiaghi, M., Bacigaluppi, M., Gallizioli, M., Gaude, E., Mari, S., … Leocani, L. (2013). Safety and efficacy of transcranial direct current stimulation in acute experimental ischemic stroke. *Stroke*, *44*, 3166–3174. http://doi.org/10.1161/STROKEAHA.113.001687

Pessiglione, M., Seymour, B., Flandin, G., Dolan, R. J., & Frith, C. D. (2006). Dopamine-dependent prediction errors underpin reward-seeking behaviour in humans. *Nature*, *442*(7106), 1042–1045. http://doi.org/10.1038/nature05051

Phillips, A. G., Vacca, G., & Ahn, S. (2008). A top-down perspective on dopamine, motivation and memory, *90*, 236–249. http://doi.org/10.1016/j.pbb.2007.10.014

Poreisz, C., Boros, K., Antal, A., & Paulus, W. (2007). Safety aspects of transcranial direct current stimulation concerning healthy subjects and patients. *Brain Research Bulletin*, *72*, 208–214. http://doi.org/10.1016/j.brainresbull.2007.01.004

Priori, a, Berardelli, a, Rona, S., Accornero, N., & Manfredi, M. (1998). Polarization of the human motor cortex through the scalp. *Neuroreport*. http://doi.org/10.1097/00001756-199807130-00020

Reis, J., Robertson, E., Krakauer, J. W., Rothwell, J., Marshall, L., Gerloff, C., … Others. (2008). Consensus:"Can tDCS and TMS enhance motor learning and memory formation?" *Brain Stimulation*, *1*(4), 363. http://doi.org/10.1016/j.brs.2008.08.001.Consensus

Reis, J., Schambra, H. M., Cohen, L. G., Buch, E. R., Fritsch, B., Zarahn, E., … Krakauer, J. W. (2009). Noninvasive cortical stimulation enhances motor skill acquisition over multiple days through an effect on consolidation. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 1590–1595. http://doi.org/10.1073/pnas.0805413106

Rioult-Pedotti, M. S., Friedman, D., Hess, G., & Donoghue, J. P. (1998). Strengthening of horizontal cortical connections following skill learning. *Nature Neuroscience*, *1*(3), 230–234. http://doi.org/10.1038/678

Robertson, E. M. (2009). From creation to consolidation: A novel framework for memory processing. *PLoS Biology*, *7*(1). http://doi.org/10.1371/journal.pbio.1000019

Romano, J. C., Howard, J. H., & Howard, D. V. (2010). One-year retention of general and sequence-specific skills in a probabilistic, serial reaction time task. *Memory (Hove, England)*, *18*(4), 427–441. http://doi.org/10.1080/09658211003742680

Rosenkranz, K., Kacar, A., & Rothwell, J. C. (2007). Differential modulation of motor cortical plasticity and excitability in early and late phases of human motor learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(44), 12058–12066. http://doi.org/10.1523/JNEUROSCI.2663-07.2007

Rossi, C., Sallustio, F., Di Legge, S., Stanzione, P., & Koch, G. (2013). Transcranial direct current

stimulation of the affected hemisphere does not accelerate recovery of acute stroke patients. *European Journal of Neurology : The Official Journal of the European Federation of Neurological Societies*, *20*, 202–4. http://doi.org/10.1111/j.1468-1331.2012.03703.x

Rossini, P. M., Barker, A. T., Berardelli, A., Caramia, M. D., Caruso, G., Cracco, R. Q., … Tomberg, C. (1994). Non-invasive electrical and magnetic stimulation of the brain, spinal cord and roots: basic principles and procedures for routine clinical application. Report of an IFCN committee. *Electroencephalography and Clinical Neurophysiology*, *91*(2), 79–92. http://doi.org/10.1016/0013-4694(94)90029-9

Rroji, O., van Kuyck, K., Nuttin, B., & Wenderoth, N. (2015). Anodal tDCS over the Primary Motor Cortex Facilitates Long-Term Memory Formation Reflecting Use-Dependent Plasticity. *Plos One*, *10*(5), e0127270. http://doi.org/10.1371/journal.pone.0127270

Sachdev, P., Fregni, F., Boggio, P., Nitsche, M. a., & Pascual-Leone, a. (2005). Transcranial direct current stimulation in developing countries [6] (multiple letters). *British Journal of Psychiatry*, *187*, 191–192. http://doi.org/10.1192/bjp.187.2.191

Saucedo Marquez, C. M., Zhang, X., Swinnen, S. P., Meesen, R., & Wenderoth, N. (2013). Task-specific effect of transcranial direct current stimulation on motor learning. *Frontiers in Human Neuroscience*, *7*(July), 333. http://doi.org/10.3389/fnhum.2013.00333

Scholz, J., Klein, M. C., Behrens, T. E. J., & Johansen-Berg, H. (2009). Training induces changes in white-matter architecture. *Nature Neuroscience*, *12*(11), 1370–1371. http://doi.org/10.1038/nn.2412

Schultz, W. (2000). Multiple reward signals in the brain. *Nature Reviews. Neuroscience*, *1*(3), 199–207. http://doi.org/10.1038/35044563

Schultz, W. (2002). Getting formal with dopamine and reward. *Neuron*, *36*, 241–263. http://doi.org/10.1016/S0896-6273(02)00967-4

Sescousse, G., Caldú, X., Segura, B., & Dreher, J. C. (2013). Processing of primary and secondary rewards: A quantitative meta-analysis and review of human functional neuroimaging studies. *Neuroscience and Biobehavioral Reviews*, *37*(4), 681–696. http://doi.org/10.1016/j.neubiorev.2013.02.002

Sharma, N., & Cohen, L. G. (2012). Recovery of motor function after stroke. *Developmental Psychobiology*, *54*, 254–262. http://doi.org/10.1002/dev.20508

Stagg, C. J., Bachtiar, V., & Johansen-Berg, H. (2011). The role of GABA in human motor learning.

Current Biology, *21*(6), 480–484. http://doi.org/10.1016/j.cub.2011.01.069

Stefan, K., Wycislo, M., Gentner, R., Schramm, A., Naumann, M., Reiners, K., & Classen, J. (2006). Temporary occlusion of associative motor cortical plasticity by prior dynamic motor training. *Cerebral Cortex*, *16*(3), 376–385. http://doi.org/10.1093/cercor/bhi116

Stinear, C. (2010). Prediction of recovery of motor function after stroke. *The Lancet Neurology*, *9*(12), 1228–1232. http://doi.org/10.1016/S1474-4422(10)70247-7

Sulzer, J. S., Chib, V. S., Hepp-Reymond, M. C., Kollias, S., & Gassert, R. (2011). BOLD correlations to force in precision grip: An event-related study. *Proceedings of the Annual International Conference of the IEEE Engineering in Medicine and Biology Society, EMBS*, (Figure 1), 2342–2346. http://doi.org/10.1109/IEMBS.2011.6090655

Takano, Y., Yokawa, T., Masuda, A., Niimi, J., Tanaka, S., & Hironaka, N. (2011). A rat model for measuring the effectiveness of transcranial direct current stimulation using fMRI. *Neuroscience Letters*, *491*(1), 40–43. http://doi.org/10.1016/j.neulet.2011.01.004

Tecchio, F., Zappasodi, F., Assenza, G., Tombini, M., Vollaro, S., Barbati, G., & Rossini, P. M. (2010). Anodal transcranial direct current stimulation enhances procedural consolidation. *Journal of Neurophysiology*, *104*(June 2010), 1134–1140. http://doi.org/10.1152/jn.00661.2009

Tennant, K. a., Adkins, D. L., Scalco, M. D., Donlan, N. a., Asay, A. L., Thomas, N., … Jones, T. a. (2012). Skill learning induced plasticity of motor cortical representations is time and age-dependent. *Neurobiology of Learning and Memory*, *98*(3), 291–302. http://doi.org/10.1016/j.nlm.2012.09.004

Tobler, P. N., Fiorillo, C. D., & Schultz, W. (2005). Adaptive coding of reward value by dopamine neurons. *Science (New York, N.Y.)*, *307*(5715), 1642–5. http://doi.org/10.1126/science.1105370

Wachter, D., Wrede, A., Schulz-Schaeffer, W., Taghizadeh-Waghefi, A., Nitsche, M. a., Kutschenko, A., … Liebetanz, D. (2011). Transcranial direct current stimulation induces polarity-specific changes of cortical blood perfusion in the rat. *Experimental Neurology*, *227*, 322–327. http://doi.org/10.1016/j.expneurol.2010.12.005

Wächter, T., Lungu, O. V, Liu, T., Willingham, D. T., & Ashe, J. (2009). Differential effect of reward and punishment on procedural learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(2), 436–443. http://doi.org/10.1523/JNEUROSCI.4132-08.2009

Wahl, a S., Omlor, W., Rubio, J. C., Chen, J. L., Zheng, H., Schröter, a, … Schwab, M. E. (2014). Neuronal repair. Asynchronous therapy restores motor control by rewiring of the rat

corticospinal tract after stroke. *Science (New York, N.Y.)*, *344*(6189), 1250–5. http://doi.org/10.1126/science.1253050

Wessel, M. J., Zimerman, M., & Hummel, F. C. (2015). Non-Invasive Brain Stimulation: An Interventional Tool for Enhancing Behavioral Training after Stroke. *Frontiers in Human Neuroscience*, *9*(May). http://doi.org/10.3389/fnhum.2015.00265

Yoon, K. J., Oh, B. M., & Kim, D. Y. (2012). Functional improvement and neuroplastic effects of anodal transcranial direct current stimulation (tDCS) delivered 1 day vs. 1 week after cerebral ischemia in rats. *Brain Research*, *1452*, 61–72. http://doi.org/10.1016/j.brainres.2012.02.062

Zaghloul, K. a, Blanco, J. a, Weidemann, C. T., McGill, K., Jaggi, J. L., Baltuch, G. H., & Kahana, M. J. (2009). Human substantia nigra neurons encode unexpected financial rewards. *Science (New York, N.Y.)*, *323*(5920), 1496–1499. http://doi.org/10.1126/science.1167342

Zeiler SR and Krakauer JW. (2012). The interaction between training and plasticity in the post-stroke brain. *Changes*, *29*(6), 997–1003. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Reward modulates changes of corticomotor excitability induced by motor practice

Orjon Rroji, Nicole Wenderoth

Abstract

Monetary reward has been shown to improve long-term retention of a new motor skill or motor adaptation in humans. The physiological substrate mediating this effect is most likely dopamine (DA), since dopaminergic neurons which are activated by reward project to primary motor cortex where they modulate the expression of synaptic plasticity. In the present study we asked whether use-dependent plasticity which is triggered by repetitively practicing the same movement is influenced when the training is performed either under rewarded or punished conditions. Twenty eight healthy young volunteers were randomly assigned to a rewarded or punished training group. All subjects practiced to flex their non-dominant thumb as fast as possible (10 blocks of 20 movements each). In the rewarded group, each trial that was executed faster than the previous one resulted in winning money. In the punished group, each trial that was executed slower than the previous one resulted in losing money. Transcranial Magnetic Stimulation (TMS) was used to determine corticomotor excitability of the non-dominant thumb flexor since previous work has shown that corticomotor excitability is typically increased after this type of training. TMS was applied at baseline before training, and approximately 0, 8, 16 and 24 min after training. Each TMS block consisted of 20 single pulses lasting approx. 6 min. Behavioural retention tests were performed 30 min after training, 1 day and 1 week later. Our main results are that mean peak velocity as well as its standard deviation followed a highly similar time course in both groups indicating that irrespective of reward/punishment subjects learned and retained the motor task equally well. Surprisingly, only the punishment group showed the typical increase of corticomotor excitability from baseline to the Post measurements (*Group x Time* interaction $F(4, 104) = 3.476$, $p < 0.05$ while the reward group exhibited even a slight decrease. Our study showed that behavioural markers of use-dependent plasticity are surprisingly insensitive to monetary reward or punishment which might result from the nature of the task. Surprisingly, training under rewarded but not under punished conditions prevented the increase of corticomotor excitability which is typically observed in this task. This finding was highly unexpected and requires further evaluation to be fully understood.

Introduction

Reward and punishment are important motivators that shape human and animal behaviour (Daw et al., 2002; Pavlov, 1927). In the context of decision making it has been shown that actions are selected to optimize reward (i.e. learning to make choices that lead to good outcome) and to minimize cost/punishment (i.e. avoiding those that lead to bad outcome). Dopamine (DA) and, particularly, dopaminergic cells in the midbrain play a key role in these learning processes (Frank et al., 2004; Pan et al., 2005; Pessiglione et al., 2007; Schultz, 2002; Zaghloul et al., 2009).

Learning and memory formation (Malenka & Bear, 2004; Martin et al., 2000) rely on activity-dependent modifications of the strength or the efficacy of synaptic transmission at pre-existing synapses (Citri & Malenka, 2008). Long term potentiation (LTP) is generally associated with strengthening synapses (for example due to repeated practice) while long term depression (LTD) is associated with weakening synapses. Previous animal research has shown that repeated practice of the same motor action triggers use-dependent plasticity in M1 so that horizontal connection of motor cortex are strengthened via LTP (Harms et al., 2008; Rioult-Pedotti et al., 2000). Moreover, it has been suggested that in humans, these cellular mechanisms of synaptic plasticity can be indirectly deduced by TMS. It has been shown that use-dependent plasticity resulting from repeatedly practicing a simple motor action (e.g. brisk thumb movements) triggers reorganisation in M1, most likely driven by an LTP-like mechanism (Bütefisch et al., 2000; Classen et al., 1998), which is reflected by an increase of corticomotor excitability (Rosenkranz et al., 2007). Additionally, LTP (but also LTD) of neurons in cortex (including M1) are modulated by dopaminergic inputs (Calabresi et al., 2007; Wickens et al., 2003) representing a potential neurophysiological link between memory formation at the one hand and practicing under rewarded versus punished condition at the other.

In line with this proposal, some recent studies have shown that reward and punishment do not only influence decision making but might also shape motor learning and memory formation. Wächter et al., (2009) demonstrated that reward (i.e. earning money for good performance) enhanced learning and retention of a serial reaction time task, while punishment (i.e. losing money for bad performance) had no influence on learning or retention when compared to a control group that received motivationally neutral cues. Using a visuo-motor tracking task, Abe et al., 2011 demonstrated enhancement of memory retention for a rewarded group of participants (winning money), while the punished group (losing money) did not show significant changes in behaviour. Whilst the previous studies used motor skill acquisition paradigms, very recently Galea et al., (2015) investigated the influence of reward/punishment on a motor adaptation task which is a model of error-based learning. They demonstrated a double dissociation so that reward and punishment have different effects on learning versus retention during motor adaptation. Furthermore, they

demonstrated that punishment (losing money) leads to faster learning but does not influence retention, whereas reward (winning money) did not influence learning gains when compared to a motivationally neutral control group but improved retention. These data reinforce the idea that independent mechanisms underpin learning and retention during motor adaptation.

In summary, previous studies used different behavioural paradigms to probe either skill acquisition (sequence learning, visuo-motor tracking) or visuo-motor adaptation under either rewarded or punished conditions. These studies showed consistently that rewarding good performance is beneficial for retention while punishing bad performance might accelerate error based learning as tested with adaptation paradigms but not necessarily other forms of motor plasticity. Even though there is growing evidence that reward/punishment influences motor memory formation as reflected at the behavioural level much less is known about the neurophysiological underpinnings of this phenomenon.

Reward processing has been strongly linked to increased firing of midbrain dopaminergic neurons that project to primary motor cortex (M1) (Descarries et al., 1987) where they facilitate synaptic plasticity and skill learning in animal models but have little influence on motor performance once a motor task has been acquired (Hosp et al., 2011; Molina-Luna et al., 2009).

Much less is known about how punishment affects motor behaviour. Recently Galea et al., (2013) showed that punishment (i.e. losing money for bad performance) influences the selection of basic kinematic parameters. Performing a simple out-and-back finger movement, the maximal acceleration as well as corticospinal excitability as measured by Transcranial Magnetic Stimulation (TMS) during movement preparation were more variable under punished than under rewarded or neutral conditions. Interestingly this effect disappeared when the dopaminergic system was manipulated via a D_2 receptor antagonist (Galea et al., 2013). The authors concluded that when participants are punished, dopamine-dependent processes influence the selection of low-level movement parameters most likely reflecting the exploration of kinematic parameters that may result in less punishing, or conversely more rewarding outcomes. Put differently, if a movement is rewarded, it is likely that the same movement will be repeated to obtain more reward (i.e. subjects exploit the actual movement solution). By contrast, if a movement causes punishment, subjects explore alternative solutions to improve performance (and thus obtain less punishment/more reward). Dopamine seems to be an important neurotransmitter that influences whether subjects switch between exploitation and exploration strategies.

Even though the above literature suggests that training under rewarded versus punished conditions might differentially influence synaptic plasticity in humans, there is virtually no neurophysiological data available to either confirm or reject this proposal. To fill the gap in knowledge we test here

whether learning under rewarded versus punished conditions leads to measurable changes of behaviour and/or corticomotor excitability in response to repetitive motor training. Subjects practiced to perform ballistic thumb movements as quickly as possible during a single training session while they were either rewarded or punished using monetary reward schemes which are known to induce powerful motivational effects (Abe et al., 2011; Wächter et al., 2009). Corticomotor excitability was measured before and after motor training using TMS. Based on previous research we expected that the motor training would result in better movement performance but also increased corticomotor excitability (Rosenkranz et al., 2007). We explored whether the behavioural and/or neural effects of training would be modulated by reward or punishment.

Material and methods

Participants

Thirty-three healthy young adult volunteers (age range 19 – 28, mean ± 22.67 ± 1.61 years) of which 17 were females and 7 left-handed (Oldfield, 1971) were recruited for this study. They had no prior experience with the motor task, were naïve regarding the purpose of the experiment and complied with standard inclusion criteria for TMS (Wassermann et al., 2008). Written inform consent was obtained from each subject before the experiment. The experimental procedure was approved by the local Ethics committee for Biomedical Science Research at the KU Leuven, Belgium, in agreement with the code of Ethics of the World Medical Association (Declaration of Helsinki)(Rickham, 1964). Five subjects were excluded from the study after TMS preparations because their rest motor threshold for TMS exceeded 70% of the maximal stimulator output. This threshold would have resulted in close to maximal stimulation intensities during the experiment bearing the risk that the TMS measurements induced discomfort which might interact with our main experimental manipulation. Therefore, 28 subjects (14 in the reward group and 14 in the punishment group) were included in the final data analyses.

Motor task and reward/punishment

Subjects were seated in a comfortable chair in front of a PC and had to perform isolated, ballistic thumb flexion movements with their non-dominant hand (Fig. 1A). The non-dominant forearm was

fixed to a wooden frame and the four fingers of the non-dominant hand were immobilized by a Velcro strap while the thumb was unconstrained and could be freely moved. A Polhemus Fastrak sensor (sampling rate of 120 Hz, spatial resolution of .0006 cm) was fixed on the nail of the moving thumb to measure 3D kinematics and to provide online feedback. This montage of the sensor location was used as previous research has shown that it is highly reproducible between sessions (Zhang et al., 2011). From the 3D kinematics, the absolute velocity was calculated by $V_i = ((X_i-X_{i-1})^2 + (Y_i-Y_{i-1})^2 + (Z_i-Z_{i-1})^2)^{1/2} / (t_i-t_{i-1})$ where X, Y and Z represent the displacement in the three dimensions, t is the time and i is the index of the current data point. For each movement, the velocity profile was displayed on the computer screen in front of the subject to provide performance feedback. Additionally, the maximum velocity was calculated and displayed for all trials, such that subjects saw how their performance changed across training.

Subjects were instructed to flex their non-dominant thumb as fast as possible. Movements were performed in blocks, each consisting of 20 movements (1 movement every 3 sec). Each block lasted 1 minute and in between blocks there was a break of 1 minute to prevent fatigue.

Visual information on the performance of the actual trial and the progression throughout the whole blocks was provided. On a screen in front the subjects could see two panels. The upper panel showed a graph in which the 3D velocity of the last thumb movement was represented (the x-axis displayed the velocity (cm/sec) and the y-axis the time (sec)). The lower part of the screen showed the amount of money the subject had won at this specific trial (i.e., the amount would either stay the same or increase if they were in the reward group or either stay the same or decrease if they were in punished group).

The following algorithm was used to provide reward/punishment based on the subject's movement performance: the rewarded group started with € 0.00 and the punished group with € 50.00 (Fig. 1B). Subjects were instructed that they would win money (rewarded group) or not lose money (punished group) when they moved faster in the actual than in the previous trial. The rewarded group received +€ 0.20 if they performed one, +€ 0.40 if they performed two or +€ 0.60 if they performed three or more trials in a row that were faster than the preceding one.

The punished group lost money when the actual trial was slower than the previous one: -€ 0.20 for one, -€ 0.40 for two, and -€ 0.60 for three or more trials that were slower than the previous one.

This reward scheme might motivate subjects to perform occasionally one very slow trial. To prevent this strategy subjects were informed that when peak velocity would suddenly drop by more than 30%, subjects would be blocked from winning money for 5 consecutive trials (rewarded group), or they would lose € 0.20 for five consecutive trials, irrespective of their performance.

Importantly, it was ensured that both groups received a comparable amount of money at the end of the experiment and the dynamics of earning/losses would be highly similar (Fig. 1 B).

Transcranial Magnetic Stimulation (TMS)

The general TMS procedure was nearly identical to the procedure described in Alaerts et al., (2009). In short, electromyograms (EMG, Mespec 8000, Mega Electronics Ltd., Kuopio, Finland) were recorded with disposable Ag-AgCl surface electrodes (Blue Sensor SP, Denmark) from the abductor pollicis brevis (APB). A belly-tendon montage was used, with one electrode placed over the middle portion of the muscle belly of the APB, and the other electrode over the APB tendon. A third reference electrode was placed on the bony part of the radius or ulna. Responses were sampled at 5000 Hz amplified (CED Power 1401 and Signal software, Cambridge Electronic Design, UK), band-pass filtered (30-1500 Hz) and stored on a PC for offline analysis. Focal TMS was performed with a 70 mm figure of eight magnetic coil connected to a Magstim 200 stimulator (Magstim, Whitland, Dyfed UK). The coil was positioned tangentially to the scalp of the non-dominant hemisphere with the handle pointing backwards at an angle of 45° away from the mid-sagittal line (Fig 1C). The subjects wore a tight fitting swimming cap covered by a grid of 1 cm^2, in order to ensure an optimal coil placement. Firstly, TMS was used to determine the so-called motor "hotspot", i.e. the position where the largest and most consistent MEPs were obtained in the APB. The APB hotspot was marked on the grid to ensure the exact replacement of the coil during the later TMS measurements. In order to obtain good TMS data, subjects were instructed to close their eyes and relax their hand muscle. This was closely monitored by the experimenter on the basis of the background EMG. The rest motor threshold (rMT), defined as the lowest stimulus intensity evoking MEP's > 50 μV in at least five out of 10 consecutive trials in the non-dominant APB, was determined to the nearest 1 % of maximum stimulator output (Rossini et al., 1994). Single pulse TMS was applied with the intensity adjusted such that MEPs with a peak-to-peak amplitude of 1 mV were evoked at baseline. This intensity was maintained for the whole experiment. MEPs were recorded in blocks of 40 stimulations with an inter-stimulus interval between 10 and 15 seconds.

Overall design

Men and women were randomly assigned to either the rewarded or punished group thus ensuring that both groups were matched for gender. We used a convenience randomization scheme which ensured that the total number of subjects per group as well as the gender ratio was matched as close as possible already during each time point of the recruitment process. The overall protocol is summarized in figure 1D. Prior to the first training session, there was a short demonstration by the experimenter which was followed by 5 warm-up trials of the subject. One block of baseline TMS measurement was run and MEPs were collected for approximately 6 min. During this time the subjects closed their eyes and were asked to relax their hand muscle which was closely monitored on the basis of the background EMG (bgEMG). Right after the collection of baseline MEPs the subjects were prepared for the behavioural task, by removing the EMG electrodes. Ten practice blocks were executed (train1 ... train10) each block consisting of 20 flexion movements (1 movement every 3 s). Each block lasted 1 min in total and was followed by a 1 min break to prevent fatigue. The total training session lasted 20 min (corresponding to 200 flexion movements) and the subject was either rewarded for good performance or punished for bad performance.

After the behavioural task the subjects were prepared to receive 4 more blocks of post-training TMS which was separated by breaks of approximately 1 minute. At the end of the last TMS block the EMG electrodes were removed and subjects performed a retention test (RT–D1) consisting of 1 block of 20 flexion movements. Additional retention tests were performed the day after (RT–D2) and one week later (RT–D7) each consisting of 3 blocks of 20 flexion movements, no TMS measurements were taken. During the whole experiment, the experimenter administering the TMS measurements was blinded as to whether subject belonged to the rewarded or punished group.
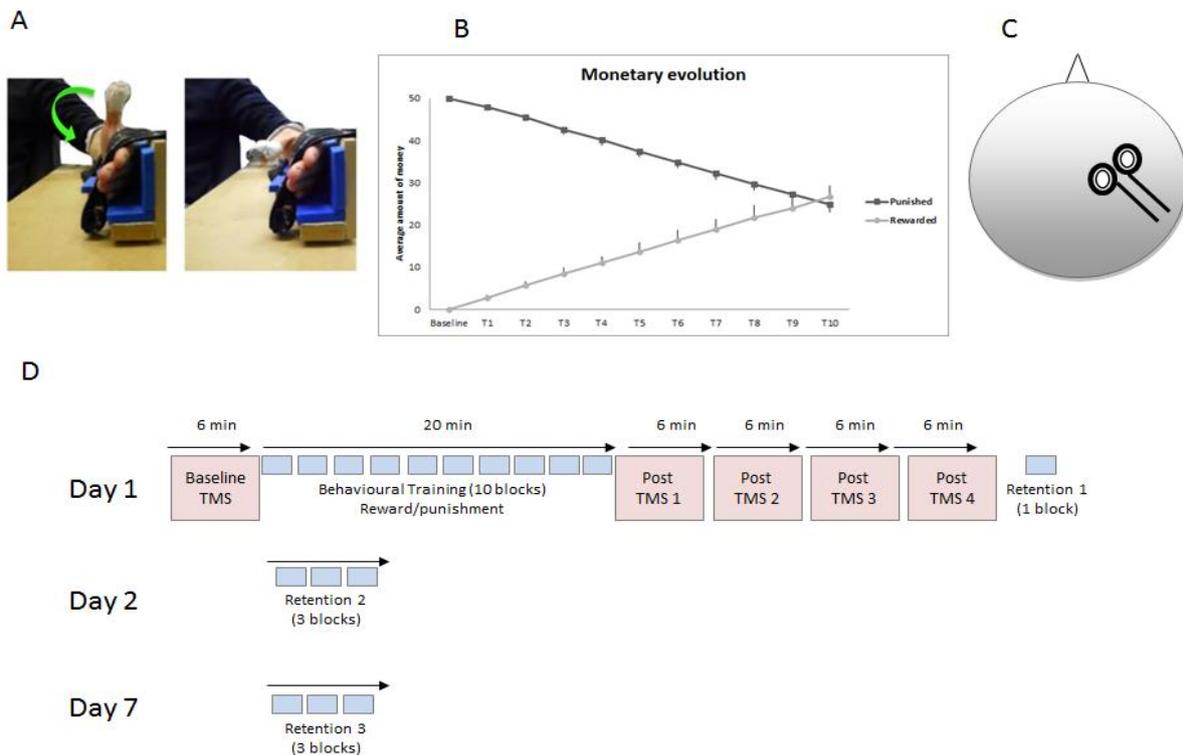
Figure 1: Experimental setup. **A)** Subjects performed discrete ballistic thumb flexion movements with the forearm and fingers fixated. **B)** Monetary evolution of rewarded and punished groups. Rewarded group (grey colour) starts the performance at 0 euro and the punished group (black colour) starts the performance at 50 euro. Y-axis: average amount of money; X-axis: training sessions. Data are shown as StD. **C)** Focal TMS positioned tangential to the scalp of the non-dominant hemisphere. **D)** General experimental design.

Data analyses

Behavioural data: The peak velocity (PeakV), i.e. the maximal 3D velocity within the 2 sec movement window was extracted for every single trial and averaged within each block. In order to remove inter-subject variability regarding the absolute velocity values (which is for example influenced by the length of the thumb), the average peak velocity data were normalized to the first training block (performance improvement$_{1...10}$ (%) = ((peak velocity$_{1...10}$ / train1) * 100). We also estimated performance variability by the standard deviation of the maximum peak velocity (stdPeakV) within each block.

To prevent that these behavioural estimates were confounded by extreme values within one block we removed trials where the PeakV deviated from the block mean by more than 2.5 standard deviations.

TMS data: Peak-to-Peak MEP amplitudes were determined from the EMG data of every TMS stimulation and averaged within each block. The bgEMG was quantified by calculating the root-mean-square error (RMSE) for 100 msec interval prior to the TMS stimulation (Alaerts et al., 2009). The data were excluded if bgEMG > (mean + (2.5*st.dev)) or if MEP > (Q3-Q1) x 1.5+Q3 with Q1, Q3 being the first and third quartile, respectively (Alaerts et al., 2011). 56 outliers out of 4480 trials were observed and removed from the final data analyses.

Statistics

28 subjects (14 per each group (reward/punishment)) were included in the final data analyses for behavioural training and TMS stimulation. The behavioural as well as the TMS data were subjected to Analyses of Variance for repeated measures (repeated measures ANOVA). The criterion for statistical significance was set to alpha $p < 0.05$ and *Fischer's LSD post-hoc* tests were used to analyse significant interaction effects.

Behavioural data: Data were normally distributed as tested with the Shapiro-Wilk test. A repeated measures ANOVA was performed to analyse the thumb flexion peak velocity (PeakV) as well as its variability (stdPeakV) with the between factor *Group* (reward/punished) and the within factor *Time* (training1-10, RT-D1, RT-D2, RT-D7). The analyses were performed to assess whether learning of the behavioural task was affected by the type of reward scheme. When appropriate a Greenhouse Geisser correction was applied.

TMS: For most time points, data were normally distributed (only data measured at POST-3 and POST-4 deviated from normality) and the assumption of sphericity was met. A repeated measures ANOVA was performed to analyse the MEP data with factors *Group* (rewarded/punished) and *Time* (baseline, POST-1, POST-2, POST-3, POST-4). The analyses were performed to assess whether training-induced changes in corticomotor excitability were different for the reward and punishment groups. Additionally we performed a control analysis not relying on the POST-3 and POST-4 data points. Based on previous results we expected the most important changes to occur from baseline to POST-1 we performed an additional analysis based on the % change in MEP amplitudes $\Delta MEP\% = 100*(MEP_{POST1}/MEP_{baseline}-1)$.

All results in the text are reported as mean (M) and standard deviation (STD). Error bars in the figure display the standard error of the mean (SEM).

Results

Behavioural data: Normalized PeakV increased significantly for both groups over the course of training (Fig. 2, *Time* main effect $F_{(15, 375)} = 11.216$, $p < 0.001$). A drop in performance was noticed when the subjects had to perform the first retention test (RT-D1) 30 min later. During the retention tests at day 2 (RT-D2) and one week later (RT-D7), both groups started their performance at a similar level as RT-D1 and further increased performance in the second and third block (Fig 2 & 3). Learning gains were very similar across groups; the repeated measures ANOVA revealed no significant *Time x Group* interaction $F_{(15, 375)} = .661$, $p = .822$ nor a significant *Group* main effect $F_{(1, 25)} = .175$, $p = .678$. Note that a very similar pattern of results was observed when non-normalized PeakV data were analysed and also for these data there was only a non-significant *Time x Group interaction* $F_{(16, 400)} = .844$, $p = .635$. Individual peak velocity data show an improvement of every single subject in the punishment (A) and reward (B) group due to training (Train 1 to Train 10) (Fig 4). Most of the subjects exhibited a performance decrease from the tenth training session to the first session of the second day of the retention test (Train 10 to RT D2-1). Even though there was inter-individual variability between subjects this was similar in punished (C) and rewarded (D) group (Fig 4).

The stdPeakV was slightly higher for the rewarded than for the punished group during training and also during the retention test (Fig. 3), however, this effect did not reach significance: *Group* effect $F_{(1, 23)} = 1.173$, $p = .2899$. Overall, stdPeakV showed no systematic change across time. Accordingly neither the *Time* main effect $F_{(16, 368)} = 1.431$, $p = .123$ nor the *Group x Time* interaction $F_{(16, 368)} = .9303$, $p = .534$ reached significance.
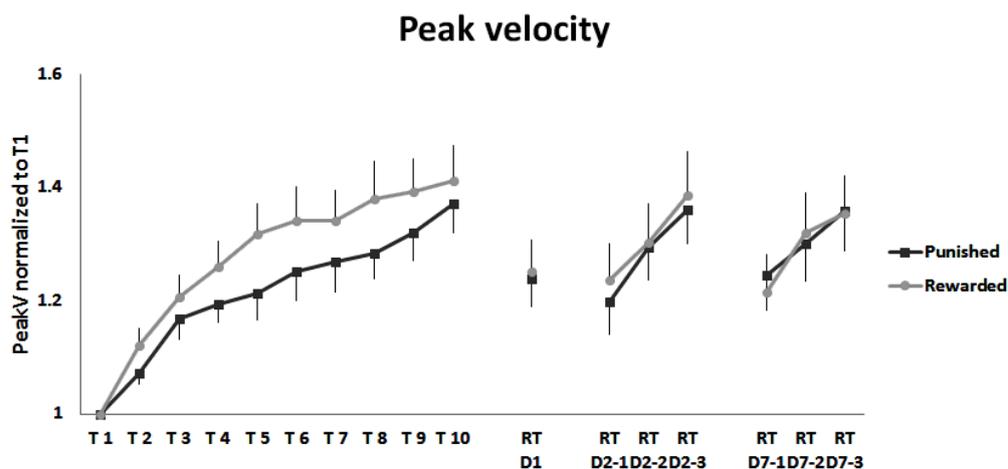

Figure 2: Peak velocity performance improvements relative to the first training block for rewarded (grey colour) and punished group (black colour). Training (T1-T10) and retention tests at day 1 (RT-D1-1), day 2 (RT-D2-1...RT-D2-3) and day 7 (RT-D7-1...RT-D7-3) were performed without stimulation. Data are shown as M ± SEM.
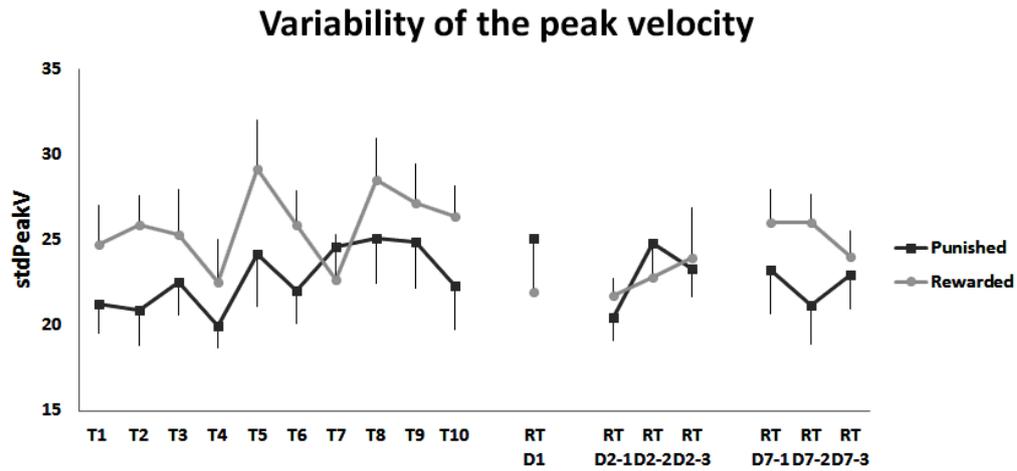
Figure 3: Performance improvements of variability of acceleration, standard deviation of peak velocity data for rewarded (grey colour) and punished group (black colour). Training (T1-T10) and retention tests at day 1 (RT-D1-1), day 2 (RT-D2-1...RT-D2-3) and day 7 (RT-D7-1...RT-D7-3) were performed without stimulation. Data are shown as M ± SEM.
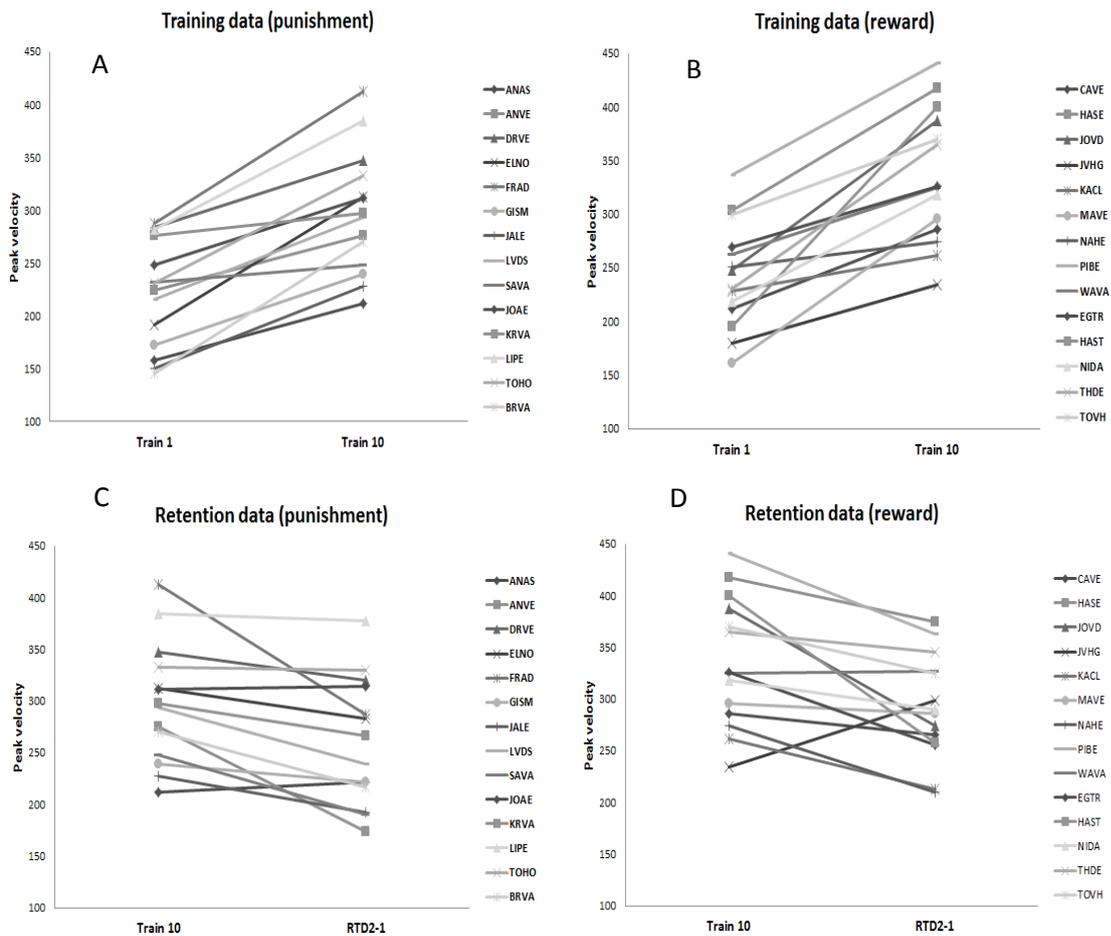

Fig 4: Individual peak velocity data of every single subject for the punishment (A) and the reward group (B) for (Train 1 to Train 10). Most of the subjects exhibited a performance decrease from the tenth training session to the first session of the second day of the retention test (Train 10 to RT D2-1). Even though there was inter-individual variability between subjects this was similar in punished (C) and rewarded group (D).

TMS data: Training induced changes in corticomotor excitability (Fig. 5) can be best understood in light of a significant *Group x Time* interaction $F(4, 104) = 3.476$, $p < 0.05$. While there was no significant group difference between the punished and rewarded group at baseline ($p = 0.15$), the punished group shows an increase of MEP amplitude from baseline to POST-1 which is then followed by a continuous increase until POST-3, followed by a small decrease at POST-4. P*ost-hoc* test showed that the baseline differed significantly ($p < 0.05$) from POST-2, POST-3, and POST-4 measurements for the punishment group (Fig. 5A). By contrast, the rewarded group exhibited a slight decrease of corticomotor excitability from baseline to POST-1 which was then followed by an increase from POST-1 to POST-3 followed by a small decrease at POST-4. P*ost-hoc* test showed no significant ($p > 0.05$) difference from baseline to either Post measurement.

Since the interaction effect was mainly driven by group differences in the baseline vs Post-1 measurement we performed a more detailed analysis of these data points. We calculated the % change in MEP amplitude measured in Post-1 relative to Baseline (ΔMEP%) and individual subject data are shown in Figure 5B. Overall there was high variability across individuals (which is not unusual for TMS measurements) but the punished group exhibited an increase in corticomotor excitability (+ 42.9 % ± 77.6) while the rewarded group exhibited a decrease (- 22.8 % ± 45.4). Even though changes just failed to reach significance when compared to 0 (single sample t-test, punishement: $t(13) = 2.07$, $p = 0.059$; reward: $t(13) = -1.87$, $p = 0.83$) there was a significant group difference (paired t-test: $t(26) = 2.74$, $p = 0.011$) suggesting that motor training under either punished or rewarded conditions differentially influenced corticomotor excitability measured immediately after training. The size of this effect was large (Cohen's $d = 1.037$).

The repeated measures Anova revealed also a significant *Time* main effect $F(4, 104) = 4.7115$, $p < 0.05$ while the *Group* effect $F(1, 26) = 1.0548$, $p > 0.05$ did not reach significance (Fig 5A).
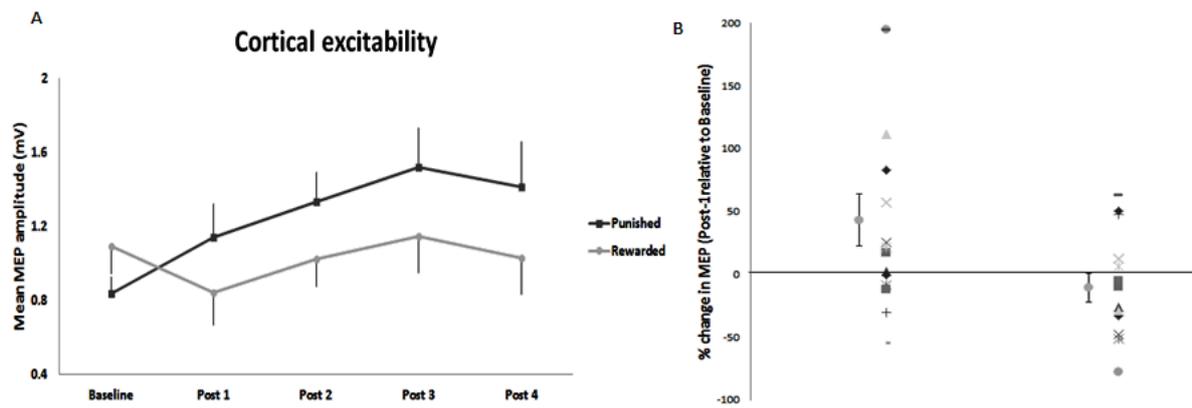
Figure 5A: Peak-to-Peak MEP amplitudes performance improvement of TMS stimulation, baseline and Post-1 … Post-4, for rewarded (grey colour) and punished group (black colour). * indicates blocks where LSD *post-hoc* tests indicate significant differences (p<0.001). Data are shown as M ± SEM. **5B:** Individual subject data. Percentage change in MEP amplitude measured in Post-1 relative to Baseline. Data are shown as M ± SEM.

Discussion

In this study we tested the influence of monetary reward versus punishment on use-dependent plasticity evoked by repetitive motor practice. This paradigm has been shown to induce neuroplastic changes in M1 which most likely result from an LTP-like mechanism and typically cause an increase of corticomotor excitability as well as behavioural improvements.

Our main results are that the reward and punishment group learned and retained the motor task equally well, based on the mean peak velocity as well as its standard deviation. Surprisingly, only the punishment group showed an increase of corticomotor excitability from baseline to Post 1 while the reward group exhibited a clear decrease.

Lack of significant influence of reward versus punishment on motor learning or retention

Previous studies showed that rewarding good performance is beneficial for the retention of different motor tasks, i.e. sequence learning (Wächter et al., 2009), visuo-motor tracking (Abe et al., 2011), and visuo-motor adaptation (Galea et al., 2015) when compared to a group of subjects that received monetary punishment or a control group that received motivationally neutral feedback. Even though our overall study design was highly similar to the previous work we could not reproduce this result (see highly similar PeakV for RT D1, RT D2 and RT D7 in figure 2).

Moreover, previous studies have shown that punishment might facilitate the learning process for error based learning paradigms, probably because exploring the movement parameter space is encouraged so that subjects find more quickly better task solutions when compared to rewarded learning or a control condition (Galea et al., 2015). Our learning data did not reproduce this effect: the punished group learned even slightly less than the rewarded group (see T1...T10 in figure 2) and also movement variability did not differ significantly between groups in the course of learning.

Thus, in summary our study revealed no evidence suggesting that the behaviour in our task was differentially influenced by rewarding or punishing subjects during training. Even though speculative, it is possible that these differential results are caused by the specific motor task used here. We will elaborate on this argument in relation to three (not mutually exclusive) hypotheses on how reward versus punishment during training might influence motor learning and retention.

First, punishment but not reward might motivate subjects to explore new movement solutions in order to improve task performance (Galea et al., 2015). However, use-dependent plasticity was triggered here by simply performing the same movement repeatedly (Classen et al., 1998; Rosenkranz et al., 2007). There is no error signal that can be used to improve performance and strategies which might facilitate learning are very limited (i.e. the only effective strategy is to fully extend the thumb thus allowing enough room for acceleration, but we instructed all subjects on this aspect prior to the experiment). Thus, the lack of a significant group difference in our study lends further support to the idea that punishment does only accelerate learning for error-related tasks.

Second, reward but not punishment are believed to influence long-term retention and Abe et al., (2011) suggest that reward activates dopaminergic neurons that project to M1 where they influence dopamine-dependent LTP that develops gradually over hours and persists for days or even weeks (Abraham, 2003). This long-lasting LTP might be essential to form memories when a new skill is acquired (like riding a bicycle). By contrast, the training performed here might have activated short-lasting plasticity which is quickly adapting and reflective of actual limb use. Therefore, it might act on a faster time scale consistent with the mechanism of early LTP (increasing excitability within 3h). Note, for example, that PeakV dropped markedly from the last training block (Figure 2, T10) to the retention test (RT D1) which were separated by approximately 30 min. In light of these findings, we tentatively suggest that our task might have activated long-lasting LTP to a much lesser degree than the tasks used in previous works.

Third, the prospect of both earning monetary reward and avoiding monetary punishment might modulate physical effort, i.e. subjects try harder to move their thumb quickly and are more persistent across the practice blocks. This hypothesis predicts that both rewarded and punished

subjects would exhibit larger improvements than subjects that receive neutral motivation. The willingness to invest physical effort might not be of large importance for sequence learning, visuo-motor skill learning or adaption (tasks that have been used in previous works). However, it might influence performance of our thumb flexion task. Unfortunately, our initial design did not include a control group that received no monetary incentives and future work might test whether both reward and punishment enhance learning relative to no monetary motivation.

Changes of corticomotor excitability in response to motor training is differently modulated by reward and punishment

Reward versus punishment during training had a differential effect on changes in corticomotor excitability when measured before and after motor practice (Figure 4). In the punished group, corticomotor excitability was significantly increased after training, an effect that has been frequently reported for this paradigm (Muellbacher et al., 2002; Rosenkranz & Rothwell, 2006). Surprisingly, no such increase of corticomotor excitability was observed for the rewarded group.

This result was highly unexpected and without replication, it is not clear whether this effect is real or just a random result. It is possible that unrelated factors which were not properly controlled in our experiment drove the group difference in corticomotor excitability. For example, our group showed recently (PhD thesis T. de Beukelaar) that the increase of corticospinal excitability in response to motor practice is strongly attenuated or even reversed to a decrease when subject spent an extended period without sleep. More specifically corticomotor excitability increased significantly less when subjects were tested in the evening than in the morning, or after a night of perturbed sleep. Nevertheless, behavioural differences were minor when compared to subjects that had sufficient sleep. In the present study we did not control this important factor and can therefore not exclude that the time of testing or the sleep pattern differed between the rewarded and the punished group.

In summary, we showed that behavioural markers of use-dependent plasticity in response to repetitive training were not differentially modulated by monetary reward or punishment. This lack of an effect might result from the specific nature of the motor task used in the present study. Alternatively, it is possible that both monetary reward and punishment influenced motivation to a similar extent, an effect we could not detect because our study did not include a control group. Moreover, we found that motor training caused an increase of corticomotor excitability but only in the punished and not in the rewarded group, a finding that requires replication and more extensive experiments in the future.

Acknowledgment

61

References

Abe, M., Schambra, H., Wassermann, E. M., Luckenbaugh, D., Schweighofer, N., & Cohen, L. G. (2011). Reward improves long-term retention of a motor memory through induction of offline memory gains. *Current Biology*, *21*, 557–562. http://doi.org/10.1016/j.cub.2011.02.030

Abraham, W. C. (2003). How long will long-term potentiation last? *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *358*(1432), 735–744. http://doi.org/10.1098/rstb.2002.1222

Alaerts, K., Swinnen, S. P., & Wenderoth, N. (2009). Is the human primary motor cortex activated by muscular or direction-dependent features of observed movements? *Cortex*, *45*, 1148–1155. http://doi.org/10.1016/j.cortex.2008.10.005

Alaerts, K., Swinnen, S. P., & Wenderoth, N. (2011). Action perception in individuals with congenital blindness or deafness: how does the loss of a sensory modality from birth affect perception-induced motor facilitation? *Journal of Cognitive Neuroscience*, *23*, 1080–1087. http://doi.org/10.1162/jocn.2010.21517

Bütefisch, C. M., Davis, B. C., Wise, S. P., Sawaki, L., Kopylev, L., Classen, J., … Bu, C. M. (2000). Mechanisms of use-dependent plasticity in the human motor cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(7), 3661–5. http://doi.org/10.1073/pnas.050350297

Calabresi, P., Picconi, B., Tozzi, A., & Di Filippo, M. (2007). Dopamine-mediated regulation of corticostriatal synaptic plasticity. *Trends in Neurosciences*, *30*(5), 211–219. http://doi.org/10.1016/j.tins.2007.03.001

Citri, A., & Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, *33*, 18–41. http://doi.org/10.1038/sj.npp.1301559

Classen, J., Liepert, J., Wise, S. P., Hallett, M., & Cohen, L. G. (1998). Rapid plasticity of human cortical movement representation induced by practice. *Journal of Neurophysiology*, *79*, 1117–1123. http://doi.org/citeulike-article-id:2143137

Daw, N. D., Kakade, S., & Dayan, P. (2002). Opponent interactions between serotonin and dopamine. *Neural Networks*, *15*(4-6), 603–616. http://doi.org/10.1016/S0893-6080(02)00052-7

Descarries, L., Lemay, B., Doucet, G., & Berger, B. (1987). Regional and laminar density of the dopamine innervation in adult rat cerebral cortex. *Neuroscience*, *21*(3), 807–824. http://doi.org/10.1016/0306-4522(87)90038-8

Frank, M. J., Seeberger, L. C., & O'Reilly, R. C. (2004). By carrot or by stick: cognitive reinforcement learning in parkinsonism. *Science (New York, N.Y.)*, *306*(5703), 1940–1943. http://doi.org/10.1126/science.1102941

Galea, J. M., Mallia, E., Rothwell, J., & Diedrichsen, J. (2015). The dissociable effects of punishment and reward on motor learning. *Nature Neuroscience*, *7*(February). http://doi.org/10.1038/nn.3956

Galea, J. M., Ruge, D., Buijink, a., Bestmann, S., & Rothwell, J. C. (2013). Punishment-Induced Behavioral and Neurophysiological Variability Reveals Dopamine-Dependent Selection of Kinematic Movement Parameters. *Journal of Neuroscience*, *33*(9), 3981–3988. http://doi.org/10.1523/JNEUROSCI.1294-12.2013

Harms, K. J., Rioult-Pedotti, M. S., Carter, D. R., & Dunaevsky, A. (2008). Transient spine expansion and learning-induced plasticity in layer 1 primary motor cortex. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *28*(22), 5686–5690. http://doi.org/10.1523/JNEUROSCI.0584-08.2008

Hosp, J. a, Pekanovic, A., Rioult-Pedotti, M. S., & Luft, A. R. (2011). Dopaminergic projections from midbrain to primary motor cortex mediate motor skill learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(7), 2481–2487. http://doi.org/10.1523/JNEUROSCI.5411-10.2011

Malenka, R. C., & Bear, M. F. (2004). LTP and LTD: An embarrassment of riches. *Neuron*, *44*(1), 5–21. http://doi.org/10.1016/j.neuron.2004.09.012

Martin, S. J., Grimwood, P. D., & Morris, R. G. M. (2000). S YNAPTIC P LASTICITY AND MEMORY: An Evaluation of the Hypothesis. *Annual Review of Neuroscience*, (Hebb 1949), 649–711. http://doi.org/10.1146/annurev.neuro.23.1.649

Molina-Luna, K., Pekanovic, A., Röhrich, S., Hertler, B., Schubring-Giese, M., Rioult-Pedotti, M. S., & Luft, A. R. (2009). Dopamine in motor cortex is necessary for skill learning and synaptic plasticity. *PLoS ONE*, *4*(9). http://doi.org/10.1371/journal.pone.0007082

Muellbacher, W., Ziemann, U., Wissel, J., Dang, N., Kofler, M., Facchini, S., … Hallett, M. (2002). Early consolidation in human primary motor cortex. *Nature*, *415*(February), 640–644. http://doi.org/10.1038/nature712

Oldfield, R. C. (1971). The assessment and analysis of handedness: the Edinburgh inventory. *Neuropsychologia*, *9*, 97–113. http://doi.org/10.1016/0028-3932(71)90067-4

Pan, W.-X., Schmidt, R., Wickens, J. R., & Hyland, B. I. (2005). Dopamine cells respond to predicted events during classical conditioning: evidence for eligibility traces in the reward-learning network. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(26), 6235–6242. http://doi.org/10.1523/JNEUROSCI.1478-05.2005

Pavlov, I. (1927). Conditioned reflexes. Routledge and Keegan Paul, London.

Pessiglione, M., Schmidt, L., Draganski, B., Kalisch, R., Lau, H., Dolan, R. J., & Frith, C. D. (2007). How the Brain Translates Money into Force, *316*(May), 1–3. Retrieved from papers://5a68cd75-9e27-4626-8d94-057f1df96060/Paper/p2150

Rickham, P. P. (1964). Human Experimentation. Code of Ethics of the World Medical Association. Declaration of Helsinki. *British Medical Journal*, *2*, 177.

Rioult-Pedotti, M. S., Friedman, D., & Donoghue, J. P. (2000). Learning-induced LTP in neocortex. *Science (New York, N.Y.)*, *290*(2000), 533–536. http://doi.org/10.1126/science.290.5491.533

Rosenkranz, K., Kacar, A., & Rothwell, J. C. (2007). Differential modulation of motor cortical plasticity and excitability in early and late phases of human motor learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(44), 12058–12066. http://doi.org/10.1523/JNEUROSCI.2663-07.2007

Rosenkranz, K., & Rothwell, J. C. (2006). Differences between the effects of three plasticity inducing protocols on the organization of the human motor cortex. *European Journal of Neuroscience*, *23*(3), 822–829. http://doi.org/10.1111/j.1460-9568.2006.04605.x

Rossini, P. M., Barker, A. T., Berardelli, A., Caramia, M. D., Caruso, G., Cracco, R. Q., … Tomberg, C. (1994). Non-invasive electrical and magnetic stimulation of the brain, spinal cord and roots: basic principles and procedures for routine clinical application. Report of an IFCN committee. *Electroencephalography and Clinical Neurophysiology*, *91*(2), 79–92. http://doi.org/10.1016/0013-4694(94)90029-9

Schultz, W. (2002). Getting formal with dopamine and reward. *Neuron*, *36*, 241–263. http://doi.org/10.1016/S0896-6273(02)00967-4

Wächter, T., Lungu, O. V, Liu, T., Willingham, D. T., & Ashe, J. (2009). Differential effect of reward and punishment on procedural learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(2), 436–443. http://doi.org/10.1523/JNEUROSCI.4132-08.2009

Wassermann, Eric M., Epstein, Charles M., Ziemann, Ulf., Walsh, Vincent., Paus, Tomas., Lisanby, S. H. (2008). The Oxford Handbook of Transcranial Stimulation. Oxford University Press 2008.

Wickens, J. R., Reynolds, J. N. J., & Hyland, B. I. (2003). Neural mechanisms of reward-related motor learning. *Current Opinion in Neurobiology*, *13*, 685–690. http://doi.org/10.1016/j.conb.2003.10.013

Zaghloul, K. a, Blanco, J. a, Weidemann, C. T., McGill, K., Jaggi, J. L., Baltuch, G. H., & Kahana, M. J. (2009). Human substantia nigra neurons encode unexpected financial rewards. *Science (New York, N.Y.)*, *323*(5920), 1496–1499. http://doi.org/10.1126/science.1167342

Zhang, X., de Beukelaar, T. T., Possel, J., Olaerts, M., Swinnen, S. P., Woolley, D. G., & Wenderoth, N. (2011). Movement observation improves early consolidation of motor memory. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(32), 11515–11520. http://doi.org/10.1523/JNEUROSCI.6759-10.2011

Chapter three

Anodal tDCS over the primary motor cortex facilitates long-term memory formation reflecting use-dependent plasticity

ORJON RROJI, KRIS VAN KUYCK, BART NUTTIN, NICOLE WENDEROTH

Abstract

Previous research suggests that anodal transcranial direct current stimulation (tDCS) over the primary motor cortex (M1) modulates NMDA receptor dependent processes that mediate synaptic plasticity. Here we test this proposal by applying anodal versus sham tDCS while subjects practiced to flex the thumb as fast as possible (ballistic movements). Repetitive practice of this task has been shown to result in performance improvements that reflect use-dependent plasticity resulting from NMDA receptor mediated, long-term potentiation (LTP)-like processes. Using a double-blind within-subject cross-over design, subjects (n=14) participated either in an anodal or a sham tDCS session which were at least 3 months apart. Sham or anodal tDCS (1 mA) was applied for 20 min during motor practice and retention was tested 30 min, 24 hours and one week later. All subjects improved performance during each of the two sessions ($p < 0.001$) and learning gains were similar. Our main result is that long term retention performance (i.e. 1 week after practice) was significantly better when practice was performed with anodal tDCS than with sham tDCS ($p < 0.001$). This effect was large (Cohen's d=1.01) and all but one subject followed the group trend. Our data strongly suggest that anodal tDCS facilitates long-term memory formation reflecting use-dependent plasticity. Our results support the notion that anodal tDCS facilitates synaptic plasticity mediated by an LTP-like mechanism, which is in accordance with previous research.

Introduction

Transcranial Direct Current Stimulation (tDCS) is a non-invasive and well-tolerated brain stimulation technique that can be applied to cortical areas [1]. tDCS modulates spontaneous neuronal network activity [2] by injecting a low amplitude direct current that passes between surface electrodes placed on the scalp. Anodal tDCS applied to the human primary motor cortex (M1) induces measurable changes in corticomotor excitability that last beyond the stimulation period, which is commonly referred to as an after-effect [3]. Numerous studies have shown that anodal tDCS over M1 combined with motor practice facilitates motor learning in healthy volunteers [4–16] even when applied during a single training session [17–19]. However, it is still not fully understood how tDCS after-effects might facilitate motor learning.

It has been hypothesized that tDCS after-effects are synaptically driven, depend on the glutamatergic system and might be mediated by a long-term potentiation (LTP)-like mechanism. These suppositions are supported by work in both human and animal models. tDCS after-effects in human are abolished when NMDA receptors are blocked [11], while facilitating NMDA receptor activity prolongs the increase in excitability caused by anodal tDCS [20]. When applied to mouse M1 slices, anodal tDCS induces long-lasting synaptic potentiation that is NMDA receptor dependent [21]. Additionally, free GABA is reduced after anodal tDCS [12] and GABAergic inhibition is released [22–24]. The reduction of GABAergic inhibition is believed to have a "gating function" to increase (glutamatergic) plasticity [25]. These effects increase the probability of LTP occurring at those synapses that are activated by behavioural processes such as motor training. Furthermore, it has also been suggested that the plasticity enhancing effect of anodal tDCS is mediated by brain derived neurotrophic factor (BDNF) dependent mechanisms which are important for structural changes at the synaptic level that promote long term consolidation [21].

While the cellular mechanisms of synaptic plasticity can be directly tested in animal models, in human they can only be indirectly inferred. One paradigm that has been shown to activate LTP-like mechanisms in human is the repeated practice of motor actions that induces neural changes known as use-dependent plasticity. For example, after several minutes of brisk thumb movements use-dependent plasticity is clearly evident [26,27]. Such training is believed to strengthen existing neural connections and to facilitate the creation of new ones within M1 [28]. Moreover, pharmacological studies have shown that its expression depends on NMDA receptor activity [29] and that effects are enhanced when GABAergic inhibition is reduced.

In summary, previous research strongly suggests that anodal tDCS over M1 acts on cellular pathways that mediate use-dependent plasticity and should therefore facilitate learning. We tested this hypothesis by applying anodal tDCS during a single training session of a ballistic thumb movement task which was followed by several retention tests that were executed 30 min, 24 hours and one week after practice had finished.

In accordance to previous work using this or similar motor tasks [27,30–33] we quantified use-dependent plasticity by changes of movement kinematics (here thumb velocity). Our underlying theoretical model is that the brain optimizes its forward command resulting in a more efficient muscle activation pattern, thus agonistic muscles are activated in a more synchronized manner (e.g. by augmenting the descending drive) while antagonists are more effectively inhibited. This will result in higher velocities/acceleration of the movement particularly for simple tasks. This theoretical model is compatible with current views suggesting that M1 neurons represent primarily kinematics rather than kinetics [34] and that training improves central representations of these movement patterns [35].

We used a double-blind within-subject cross-over design where subjects practiced ballistic thumb movements while either anodal tDCS or sham tDCS was applied during two separate sessions that were at least 3 months apart. The cross-over design was chosen to reduce the influence of inter-individual differences in ability to undergo practice related neuroplastic changes, which can vary substantially and might result from the genetic background of the individual [36] or previous motor experience [30].

Materials and methods

Participants

Eighteen young healthy volunteers were recruited for this study. Four subjects did drop out for personal reasons before performing the cross-over test and were excluded from all analyses. The remaining 14 subjects were between 18 – 29 years of age (mean age = 23 ± 7 years, 7 male). Ten subjects were right-handed (Edinburgh Handedness Inventory) [37]. None of the subjects had prior experience with the motor task and all were naïve to the purpose of the experiment. Subjects provided written informed consent prior to participation and were reimbursed. All experimental procedures were approved by the Ethics Committee for Biomedical Research at the KU Leuven

(ethics approval number: S52763) in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) [38].

Motor task

Subjects were seated in a comfortable chair and had to perform discrete ballistic thumb flexion movements with their non-dominant hand (Fig.1A). The forearm was fixed to a wooden construction and the four fingers were immobilized by a velcro strap while the thumb was unconstrained and could move freely. A Polhemus Fastrak sensor (sampling rate of 120 Hz, spatial resolution of 0.0006 cm) was fixed on the nail of the thumb to measure 3D kinematics and provide online feedback. This sensor location was used because previous research has shown that it is highly reproducible between sessions [31]. 3D kinematic data was used to calculate the absolute velocity: $V_i = \text{sqrt} \left((X_i - X_{i-1})^2 + (Y_i - Y_{i-1})^2 + (Z_i - Z_{i-1})^2 \right) / (t_i - t_{i-1})$ where X, Y and Z represent displacement in three dimensions, t the time and i the index of the current data point. For each movement the velocity profile was displayed on a computer screen in front of the subject to provide performance feedback. The maximum velocity was also displayed for each trial and continuously updated providing subjects with an indication of how their performance changed across training.

tDCS

Transcranial direct current stimulation (tDCS) was delivered by a battery driven constant current stimulator (HDC stim part of HDC kit, Medical device CE 0068, Newronika s.r.l. Milan – Italy) which was connected to two rubber electrodes enclosed in saline soaked sponges (Fig. 1B). The anode (5 x 5 cm) was located over the hand area of the M1, which was localized with transcranial magnetic stimulation (TMS). The general TMS procedure was nearly identical to that described in Alaerts et al., [39]. In short, electromyograms (EMG, Mespec 8000, Mega Electronics Ltd., Kuopio, Finland) were recorded with disposable Ag-AgCl surface electrodes (Blue Sensor SP, Denmark) from the abductor pollicis brevis (APB). Focal TMS was performed with a 70mm figure of eight magnetic coil connected to a Magstim 200 stimulator (Magstim, Whitland, Dyfed UK). The coil was positioned tangential to the scalp of the non-dominant hemisphere with the handle pointing backward at an angle of 45º away from the mid sagittal line. TMS was used to determine the so-called motor "hotspot", i.e. the position where the largest and most consistent MEPs were obtained in the APB. The APB hotspot was marked on the scalp and the centre of the anodal electrode was positioned over this point. The

average hotspot position was 5.2 ± 0.8 cm lateral to the midline and 0.9 ± 1.1 cm anterior to the intraural line. The cathode (11 x 9 cm) was located on the ipsilateral shoulder (extracephalic placement). We did not test TMS in 4 subjects because of technical problems (malfunctioning and repair of stimulator) and we placed the electrode 5 cm laterally from the cortex and 1 cm anterior to the intraaural line.

In the anodal tDCS condition the current was ramped up to 1.0 mA over 12 s and then applied at this intensity continuously for 20 min. In the sham tDCS condition the same ramp up procedure was applied, but the current was ramped down after 12 s (sham tDCS).
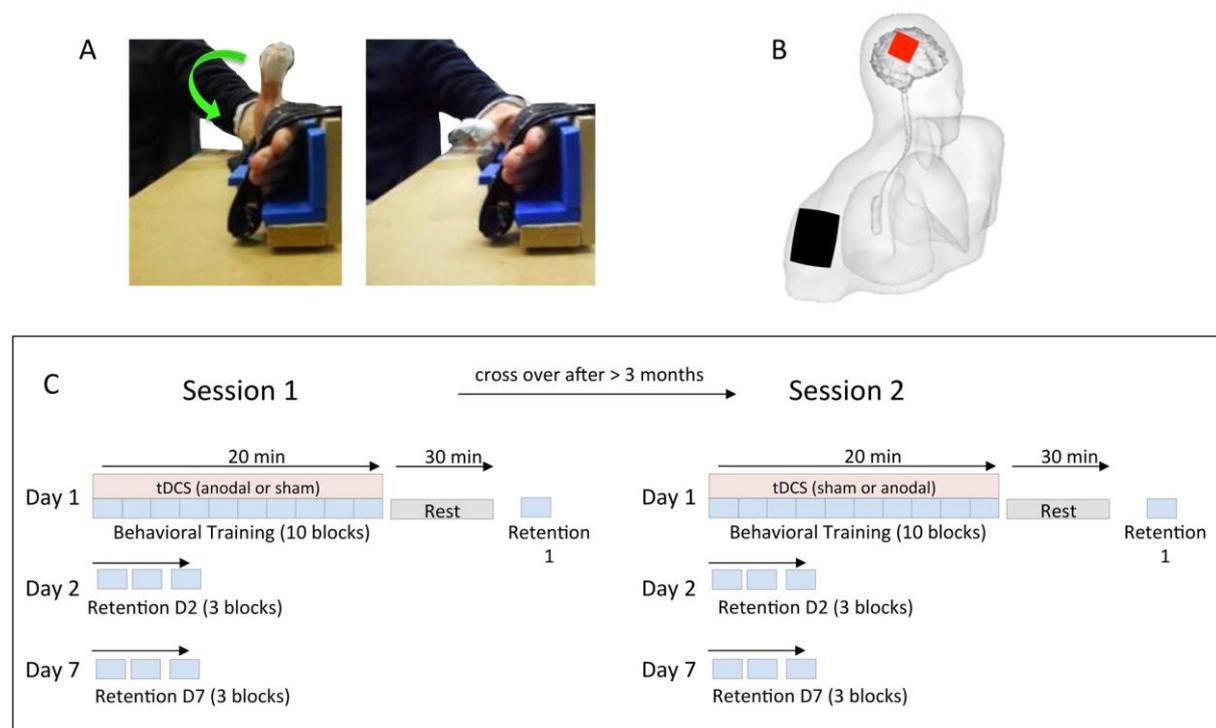

Fig. 1: Experimental Setup. A) Subjects performed discrete ballistic thumb flexion movements with the forearm and fingers fixated. B) Constant current stimulation was delivered with the anode (red) placed over the M1 contralateral to the moving thumb and the cathode (black) over the ipsilateral shoulder. C) General experimental design.

Overall design

We employed a cross-over design with all subjects participating in anodal tDCS and sham tDCS sessions (order counterbalanced across subjects) which were at least 12 weeks apart (Fig.1C). Both subjects and the experimenter were blinded as to which stimulation was applied.

At the beginning of each session there was a short demonstration by the experimenter that was followed by 5 warm-up trials. Ten practice blocks were then executed (train 1…train 10) each consisting of 20 flexion movements (1 trial every 3 s). One practice block lasted 1 min in total and was followed by a 1 min break to prevent fatigue. The total training session lasted 20 min (corresponding to 200 flexion movements) and during this time either anodal tDCS or sham tDCS was administered. After training the tDCS electrodes were removed and subjects rested for 30 min. A retention test (RT-D1) was then performed consisting of 1 block of 20 flexion movements. Additional retention tests were performed the following day (RT-D2) and one week (RT-D7) later, each consisting of 3 blocks of 20 flexion movements (S1 File).

At the end of the experiment subjects were debriefed. None reported suffering serious headaches, nausea or pain. Even though some subjects reported an initial tingling sensation they perceived no difference between the two sessions, which was likely due to the fact that sessions were at least 3 months apart.

Statistics

Since a cross-over design was used, i.e. subjects had to come in the lab twice to perform the motor training task, we first examined the influence of session-effects on performance improvement. Peak velocities were averaged within each block. All blocks were normally distributed (Shapiro-Wilk test, $p > 0.055$) and the assumption of sphericity was met (Mauchly's test of sphericity). We tested for potential session-effects by entering these data into an analysis of variance for repeated measurements (repeated measures ANOVA) with the within-subjects factors *session* (1, 2) and *block* (train1…train10).

Next we tested the effect of anodal versus sham tDCS on use-dependent plasticity. For each session performance was normalized to the first training block (performance improvement$_{1…10}$ (%) = ((peak velocity$_{1…10}$ / train1) * 100) (S1 File). Data were normally distributed, except for one block ($p = 0.042$ for train 5 of the anodal tDCS session), and the assumption of sphericity was met. The % performance improvement data were entered into a repeated measures ANOVA with the within-subjects factors *stimulation* (anodal tDCS, sham tDCS) and *block* (train2…train10, RT-D1-1, RT-D2-1… RT-D2-3, RT-D7-

1… RT-D7-3), and the between-subjects factor *order* (anodal-sham, sham-anodal). The alpha level was α = 0.05 and Fischer's LSD *post-hoc* tests were used to analyse significant interaction effects. Finally, for significant *stimulation* x *block* interaction effects Cohen's d (effect size for dependent measurements) was calculated. Further details are described in the results section. All results in the text are reported as mean (M) and standard deviation (STD). Error bars in the figures display the standard error of the mean (SEM).

Results

Training resulted in a reliable increase in thumb flexion peak velocity which was observed for each session (Fig. 2; main effect of *block* $F(16, 208) = 19.20$, $p < 0.0001$; note that for each session data was collapsed across anodal tDCS and sham tDCS conditions). Not surprisingly, overall peak velocities were significantly higher in the second than the first session (main effect of *session*: $F(1, 12) = 11.30$, $p < 0.005$). Importantly, the learning gains (indicating that use-dependent plasticity took place) were similar across sessions (*session* x *block* interaction: $F(16, 208) = 0.81$, $p = 0.675$), i.e. we found no statistical evidence indicating that subjects learned more in the first than the second session (or vice versa).
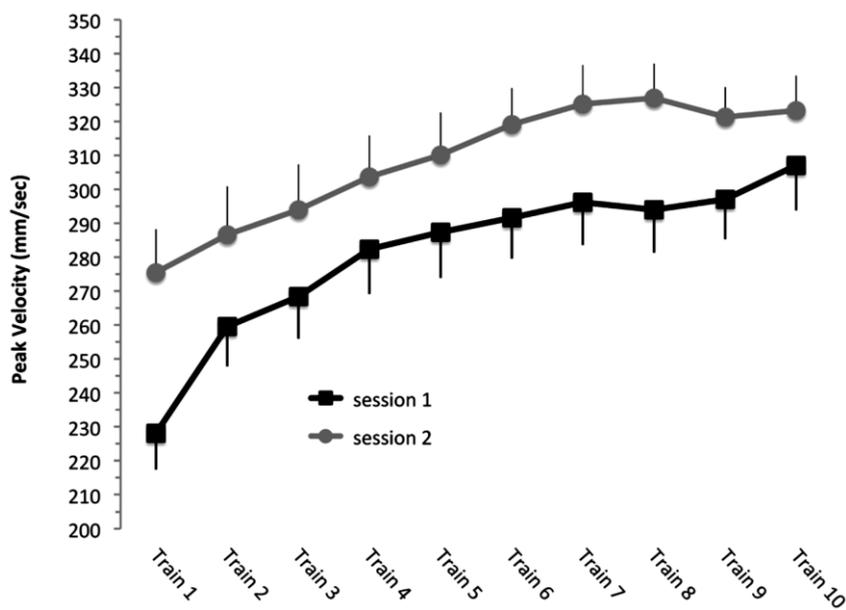

Fig. 2: Order effects. Peak velocity data of the practice blocks (train1…10) performed in session 1 (black squares) and session 2 (grey circles). Note that when data are collapsed across anodal tDCS and sham tDCS conditions peak velocity was generally higher in the second session, but the extent of improvement over the course of learning was similar. Data are shown as M ± SEM.

Next we investigated whether training with anodal tDCS influenced performance gains or retention differently than training with sham tDCS. Performance improvements relative to the first practice block (train1) are shown in Fig. 3. Learning occurred in both sessions (main effect of *block*: $F(15,180) = 12.89$, $p < 0.0001$) and differences in learning gains during training (solid symbols) were minor when compared between anodal tDCS and sham tDCS sessions (main effect of *stimulation*: $F(1, 12) = 1.598$, $p = 0.230$). However, during the retention tests (performed without stimulation), performance in the two sessions started to differ. RT performance following training with anodal tDCS was better than RT performance following training with sham tDCS, an effect that reached significance at RT-D7 (*block* x *stimulation* interaction: $F(15, 180) = 3.21$, $p < 0.001$).

We further investigated whether the effect of anodal tDCS during training on retention performance one week later (RT-D7) was consistent across individuals. Performance savings/gains for the anodal tDCS (**Δ**anodal) and sham tDCS (**Δ**sham) sessions were calculated at the single subject level by subtracting the average % performance improvement at the end of training (i.e. the average of practice blocks train8…train10) from the average % performance improvement at RT-D7 (i.e. the average of RT-D7-1…3). Fig. 4 shows that all but one participant had larger savings/gains when they trained with anodal tDCS than when they trained with sham tDCS. Note, however, that there were large individual differences whether participants exhibited performance gains (i.e. better performance at RT-D7 than at train8…train10) or losses (i.e. worse performance at RT-D7 than at train8…train10). Effect size was calculated by dividing the mean of individual differences between gains/losses of the anodal tDCS versus sham tDCS session by the standard deviation, i.e.

$$Cohen's\ d = \frac{mean(\Delta anodal_i - \Delta sham_i)}{stdev(\Delta anodal_i - \Delta sham_i)}$$

with *i* indexing all individual subjects, yielding a Cohen's d of 1.01, i.e. anodal tDCS had a *large* effect on retention performance.
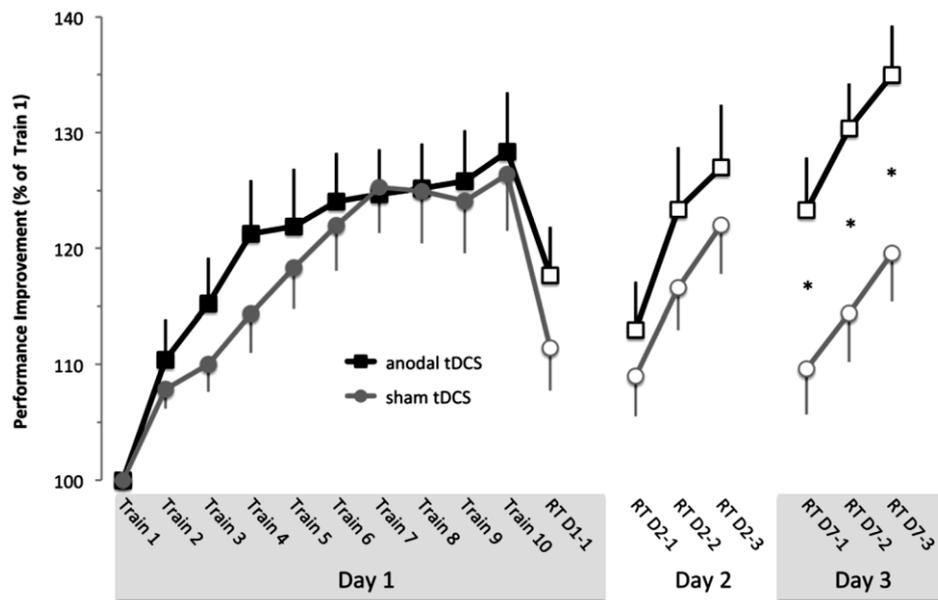
Fig. 3: Stimulation effects. Performance improvements relative to the first training block for the anodal tDCS session (black squares) and the sham tDCS session (grey triangles). Training was performed while stimulation was applied (filled symbols), while retention tests at day 1 (RT-D1-1), day 2 (RT-D2-1…RT-D2-3) and 7 (RT-D7-1...RT-D7-3) were performed without stimulation (open symbols). * indicates blocks where LSD post hoc tests indicate significant differences of anodal tDCS versus sham stimulation (p < 0.001). Data are shown as M ± SEM.

There was also a significant *stimulation* x *order* interaction effect (F(1,12) = 7.44, p = 0.018) that is the result of subjects in the anodal-sham group exhibiting larger overall performance improvements in the first than in the second session, while subjects in the sham-anodal group improved more in the second than in the first session (Fig. 5). This confirms that differences in performance improvement between sessions resulted from the stimulation condition rather than from unspecific order effects.
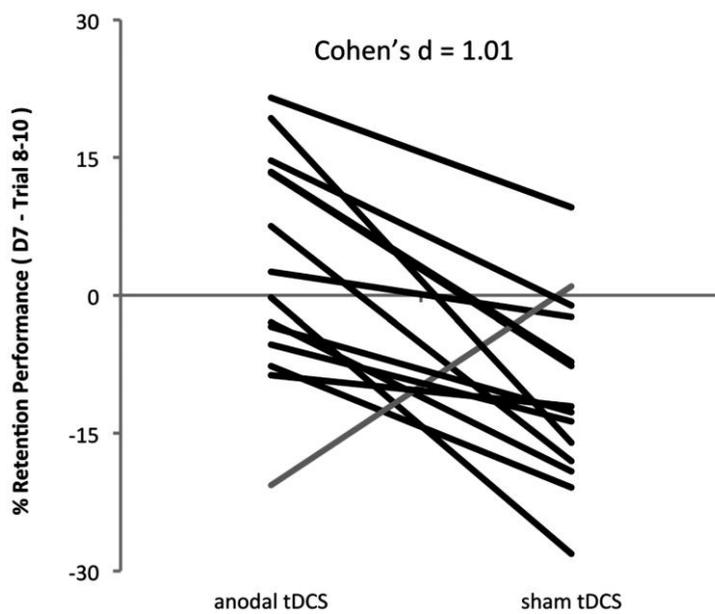
Fig. 4: Individual subject data. Individual subject data showing gains/savings measured during the retention test at day 7 compared to performance at the end of training (i.e. average performance at RT-D7-1...3 minus average performance at train8...10). Individuals exhibiting the same trend as the group average are shown in black. Only one subject (grey) exhibited better retention performance after practice with sham tDCS than after practice with anodal tDCS.
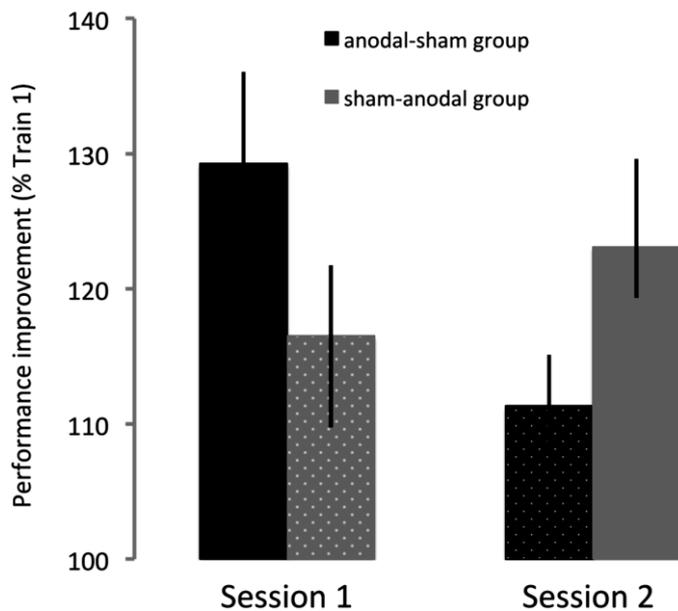
Fig. 5: Significant *session x order* interaction. Individuals in the anodal – sham group (black, n=7) exhibited larger performance improvements in the first than in the second session. By contrast, individuals in the sham – anodal group (grey, n=7) exhibited smaller performance improvements in the first than in the second session. This finding lends further

support to the observation that practice with anodal tDCS (solid bars) facilitated learning in comparison to sham tDCS (dotted bars). Data are shown as M ± SEM.

Discussion

In the present study we induced use-dependent plasticity through repetitive motor practice and used this phenomenon as a model to study the influence of anodal versus sham tDCS on LTP-like synaptic plasticity in human M1. Our main result is that retention performance was significantly better when anodal tDCS was applied during training when compared to training with sham tDCS. Significance was only reached when retention was tested one week after training, however, the size of this effect was large (Cohen's d = 1.01) and consistent across subjects (13 out of 14 subjects followed the trend at the group level).

Our finding that anodal tDCS facilitated motor memory formation, but that its beneficial effect was mainly expressed in a retention test, is in line with previous work [9,18,40–46]. In particular Reis at al., [41] observed that when tDCS was applied to M1 during visuomotor adaptation, performance benefits were only found 3 h after the end of training. Similar to the present study they also found that no additional performance gains were observed when retention was tested after a single night of sleep, suggesting that the beneficial effect of anodal tDCS on memory consolidation is not sleep dependent. Moreover, Reis et al., [41] reported that beneficial effects were not found when stimulation was applied after practice, suggesting that the simultaneous application of anodal tDCS and practice triggers subsequent processes important for motor memory formation. Our study confirms and extends this research by demonstrating that anodal tDCS modulates the long-term effects of use-dependent plasticity, a phenomenon that is believed to be mediated by strengthening synapses via a LTP-like process [26,27,29].

One has to note, however, that polarity specific effects of tDCS on neuroplasticity might differ across brain areas. For example, Peters et al., [47] demonstrated that applying anodal tDCS over the primary visual cortex during perceptual learning blocks rather than facilitates memory consolidation. Thus, the plasticity enhancing effect of anodal tDCS reported here might be specific to the motor cortex.

In our study it is surprising that the strongest effects were found when retention was tested one week after training. Note that the D2 retention test consisted of 3 practice blocks which resulted in highly significant performance improvements regardless of whether initial training was performed with anodal or sham tDCS (separate repeated measures ANOVA with the factor *block* (D2-RT1...3:

$F(2,11) \geq 13.82$, $p \leq 0.001$). In other words, D2 was not only a retention test but also a second training session. A clear dissociation in performance between anodal and sham tDCS training only occurred after the D2 practice blocks were finished and retention was tested again at D7. More specifically, following the anodal tDCS session the performance level reached at the end of D2 was largely maintained when long term retention was tested at D7, while it was nearly completely forgotten when initial training occurred with sham tDCS. This pattern of results suggests that combining anodal tDCS with training during D1 might have upregulated plasticity mediating mechanisms for approximately 24 hours, which in turn led to subsequent practice at D2 resulting in better long term memory formation. Indirect support for this proposal comes from Monte-Silva et al., [48] who showed that when anodal tDCS was applied at rest (i.e. during two 13 min sessions separated by 3 or 20 min), corticomotor excitability in M1 remained elevated for more than 24 hours after stimulation. Thus, in principle, tDCS effects can outlast stimulation by more than 24 hours, however, future research is required to confirm whether a similar principle is also applicable when anodal tDCS is combined with motor training because the interaction between plasticity inducing brain stimulation and training is complex, non-additive [49] and might be influenced by homeostatic principles [50].

In the present study we used a within subject cross-over design which is advantageous because it controls for inter-individual variability in ability to improve performance due to practice, which can be large [36] and might mask the modulatory effects of tDCS. Additionally, this design can be used to quantify the effect of anodal tDCS on memory formation at the level of the individual. Interestingly, in our study all but one subject had better retention performance in the anodal tDCS session than in the sham tDCS session, and the effect was large (Cohen's d = 1.01) indicating that our sample responded very consistently to the experimental manipulation.

Our study also has several limitations. First, based on previous work one would expect that the effect of tDCS on motor memory formation might be more reliably measured when it accumulates over multiple sessions [40,41,51,52]. We used a single practice session that was followed by several retention tests because we were concerned that too much training would lead to highly automatized performance during the first session, which would lead to either no or significantly less learning gains during the second session. With our rather short practice period we were indeed able to show that performance was generally better in the second than in the first session, but that learning gains were comparable and did not differ significantly. Second, we used a relatively simple motor task that might, in theory, cause ceiling effects in the response to anodal tDCS [53,54]. It is possible that the tDCS effect size is even bigger for more complex skills. Finally, we did not stimulate a control region, and thus cannot provide any insight into the anatomical specificity of the effect.

In summary our data strongly suggest that anodal tDCS facilitates long term memory formation reflecting use-dependent plasticity in the motor cortex. We used this task because previous research has convincingly demonstrated that performance changes reflect synaptic plasticity mediated by an LTP-like mechanism and, in line with this work, our results suggest that anodal tDCS might facilitate these processes.

Supporting Information

S1 File. Normalized performance data (tab separated txt file).

name	st sess	xperiment	Train 1	Train 2	Train 3	Train 4	Train 5	Train 6	Train 7	Train 8	Train 9	Train 10	RT D1-1		RT D2-1	RT D2-2	RT D2-3		RT D7-1	RT D7-2	RT D7-3
			anode	anode	anode	anode	anode	anode	anode	anode	anode	anode	anode		anode	anode	anode		anode	anode	anode
CATE (m)	anode	exp1	100	134.724	132.628	141.221	134.756	135.304	144.285	133.351	129.401	143.715	133.58		137.047	147.86	157.412		148.215	144.862	153.277
DIPE (m)	anode	exp1	100	131.579	149.707	159.093	164.382	141.687	133.696	133.373	147.168	162.537	151.452		140.08	161.651	165.342		157.441	160.416	147.808
DYBE (m)	anode	exp1	100	113.473	125.955	122.297	116.922	126.754	126.239	129.406	118.953	128.675	113.038		113.501	125.797	127.703		115.467	117.737	120.841
FLVH (m)	anode	exp1	100	114.304	116.994	117.844	122.308	133.609	134	134.927	133.877	131.435	110.607		99.9158	112.577	116.783		107.198	133.298	150.994
KAJE (f)	anode	exp1	100	101.959	105.939	107.149	109.75	109.866	109.495	104.452	109.913	115.069	104.144		90.1546	100.276	105.232		105.56	112.336	110.856
MAHI (f)	anode	exp1	100	105.724	106.152	114.158	109.039	108.079	107.964	119.298	127.511	122.575	121.205		113.542	134.828	136.015		114.121	125.192	137.804
MAPA (m)	anode	exp1	100	122.781	125.08	143.472	155.408	159.281	153.585	159.847	163.241	163.241	129.064		130.013	137.514	132.289		133.315	142.092	148.963
EGSE (f)	sham	exp1	100	85.381	95.3685	106.482	113.633	126.259	126.907	127.933	126.31	132.005	112.744		119.327	107.574	111.476		113.596	123.649	122.926
GIVE (m)	sham	exp1	100	108.752	114.528	121.642	115.005	123.279	125.615	123.362	119.669	130.401	117.108		105.176	116.224	122.156		113.397	122.877	126.906
GRVE (f)	sham	exp1	100	103.559	106.906	121.399	119.556	118.756	120.14	122.745	122.82	118.307	108.265		105.367	105.248	107.322		110.213	115.876	121.707
KAPO (f)	sham	exp1	100	109.429	117.984	129.17	125.898	115.714	127.779	124.701	125.777	107.487	107.522		99.2217	106.377	110.46		133.615	132.254	136.021
SISM (f)	sham	exp1	100	113.088	115.168	113.514	115.977	120.717	122.957	119.236	116.027	111.426	107.784		106.358	119.824	123.374		132.513	137.627	140.996
TIVS (f)	sham	exp1	100	103.997	105.711	106.124	108.236	122.639	114.702	122.475	128.089	134.37	138.411		125.118	151.197	156.335		137.867	145.483	159.424
WOHO (m)	sham	exp1	100	97.132	94.9858	94.0274	95.4908	94.7408	98.1821	97.463	92.4725	96.0033	92.3704		96.8156	100.162	105.931		103.78	111.328	111.122

name	st sess	xperiment	Train 1	Train 2	Train 3	Train 4	Train 5	Train 6	Train 7	Train 8	Train 9	Train 10	RT D1		RT 1 D2	RT 2 D2	RT 3 D2		RT 1 D7	RT 2 D7	RT 3 D7
			sham	sham	sham	sham	sham	sham	sham	sham	sham	sham	sham		sham	sham	sham		sham	sham	sham
CATE (m)	anode	exp1	100	104.198	110.258	109.865	108.703	111.789	112.445	113.852	106.494	113.59	100.648		97.4974	96.992	106.878		98.2446	104.794	107.807
DIPE (m)	anode	exp1	100	102.255	110.714	105.624	115.813	118.164	112.629	112.011	120.371	123.081	100.315		107.733	113.342	119.438		97.9988	98.7092	104.659
DYBE (m)	anode	exp1	100	108.957	106.07	105.139	109.361	115.468	117.887	119.788	115.427	114.99	101.434		101.777	108.638	117.603		85.4318	102.561	99.4476
FLVH (m)	anode	exp1	100	106.96	106.613	111.581	115.508	125.422	129.179	128.198	127.045	132.917	115.486		105.819	115.97	124.563		110.724	108.628	111.335
KAJE (f)	anode	exp1	100	102.973	109.246	116.519	121.548	128.269	130.006	141.096	130.222	138.962	122.116		114.005	119.258	130.718		101.691	104.151	120.075
MAHI (f)	anode	exp1	100	103.367	98.5088	105.289	106.574	108.171	115.767	118.62	117.128	110.105	96.3385		115.572	123.237	135.198		111.354	107.819	119.561
MAPA (m)	anode	exp1	100	106.51	105.216	106.685	112.792	106.436	111.605	107.475	105.323	99.8643	103.965		97.1233	100.127	97.6405		104.731	103.378	107.463
EGSE (f)	sham	exp1	100	111.393	117.972	136.315	136.12	136.72	140.943	154.861	143.123	157.164	136.325		121.53	130.704	130.225		136.187	139.419	143.357
GIVE (m)	sham	exp1	100	115.437	118.98	129.649	123.911	127.685	129.859	122.742	125.397	133.001	119.61		107.672	123.754	128.345		104.932	118.103	119.973
GRVE (f)	sham	exp1	100	113.025	129.715	128.668	144.036	148.535	151.545	149.36	154.091	155.559	139.853		127.099	136.147	136.084		131.769	139.932	146.122
KAPO (f)	sham	exp1	100	111.228	99.3187	103.364	102.95	126.653	128.838	119.704	129.478	124.172	111.555		114.037	125.666	112.879		114.584	127.273	128.177
SISM (f)	sham	exp1	100	98.0151	105.605	111.029	113.939	112.767	115.491	114.8	108.901	113.159	106.649		103.533	120.483	127.936		115.148	120.644	129.73
TIVS (f)	sham	exp1	100	122.291	120.553	133.781	140.976	144.525	151.793	147.512	152.999	147.214	109.329		131.104	129.489	149.891		128.288	134.204	137.16
WOHO (m)	sham	exp1	100	103.655	101.31	97.3994	104.482	97.1956	105.682	99.131	101.277	105.497	96.5229		81.215	88.8031	90.9454		93.4013	91.7819	99.0686

Acknowledgments

Reference

1.   Paulus W. Transcranial direct current stimulation (tDCS). Suppl Clin Neurophysiol. 2003. pp. 249–254. doi:10.1016/j.neuropsychologia.2015.02.002

2.   Nitsche MA, Cohen LG, Wassermann EM, Priori A, Lang N, Antal A, et al. Transcranial direct current stimulation: State of the art 2008. Brain Stimul. 2008; 1: 206–223. doi:10.1016/j.brs.2008.06.004

3.   Nitsche MA, Paulus W. Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. J Physiol. 2000; 527 Pt 3: 633–639. doi:10.1111/j.1469-7793.2000.t01-1-00633.x

4.   Brasil-Neto JP. Learning, memory, and transcranial direct current stimulation. Front Psychiatry. 2012; 3: 1–4. doi:10.3389/fpsyt.2012.00080

5.   Orban de Xivry J-J, Shadmehr R. Electrifying the motor engram: effects of tDCS on motor learning and control. Exp Brain Res. 2014; 232: 3379–3395. doi:10.1007/s00221-014-4087-6

6.   Schambra HM, Abe M, Luckenbaugh DA, Reis J, Krakauer JW, Cohen LG. Probing for hemispheric specialization for motor skill learning: a transcranial direct current stimulation study. J Neurophysiol. 2011; 106: 652–661. doi:10.1152/jn.00210.2011

7.   Boggio PS, Castro LO, Savagim EA, Braite R, Cruz VC, Rocha RR, et al. Enhancement of non-dominant hand motor function by anodal transcranial direct current stimulation. Neurosci Lett. 2006; 404: 232–236. doi:10.1016/j.neulet.2006.05.051

8.   Vines BW, Nair D, Schlaug G. Modulating activity in the motor cortex affects performance for the two hands differently depending upon which hemisphere is stimulated. Eur J Neurosci. 2008; 28: 1667–1673. doi:10.1111/j.1460-9568.2008.06459.x

9.   Galea JM, Celnik P. Brain polarization enhances the formation and retention of motor memories. J Neurophysiol. 2009; 102: 294–301. doi:10.1152/jn.00184.2009

10.   Tanaka S, Hanakawa T, Honda M, Watanabe K. Enhancement of pinch force in the lower leg by anodal transcranial direct current stimulation. Exp Brain Res. 2009; 196: 459–465. doi:10.1007/s00221-009-1863-9

11. Nitsche MA, Schauenburg A, Lang N, Liebetanz D, Exner C, Paulus W, et al. Facilitation of implicit motor learning by weak transcranial direct current stimulation of the primary motor cortex in the human. J Cogn Neurosci. 2003; 15: 619–626. doi:10.1162/089892903321662994

12. Stagg CJ, Best JG, Stephenson MC, O'Shea J, Wylezinska M, Kincses ZT, et al. Polarity-sensitive modulation of cortical neurotransmitters by transcranial stimulation. J Neurosci. 2009; 29: 5202–5206. doi:10.1523/JNEUROSCI.4432-08.2009

13. Antal A, Nitsche MA, Kincses TZ, Kruse W, Hoffmann KP, Paulus W. Facilitation of visuo-motor learning by transcranial direct current stimulation of the motor and extrastriate visual areas in humans. Eur J Neurosci. 2004; 19: 2888–2892. doi:10.1111/j.1460-9568.2004.03367.x

14. Orban de Xivry JJ, Marko MK, Pekny SE, Pastor D, Izawa J, Celnik P, et al. Stimulation of the human motor cortex alters generalization patterns of motor learning. J Neurosci. 2011; 31: 7102–7110. doi:10.1523/JNEUROSCI.0273-11.2011

15. Pavlova E, Kuo MF, Nitsche MA, Borg J. Transcranial direct current stimulation of the premotor cortex: Effects on hand dexterity. Brain Res. 2014; 1576: 52–62. doi:10.1016/j.brainres.2014.06.023

16. Karok S, Witney AG. Enhanced motor learning following task-concurrent dual transcranial direct current stimulation. PLoS One. 2013; 8: 1–10. doi:10.1371/journal.pone.0085693

17. Kantak SS, Mummidisetty CK, Stinear JW. Primary motor and premotor cortex in implicit sequence learning - Evidence for competition between implicit and explicit human motor memory systems. Eur J Neurosci. 2012; 36: 2710–2715. doi:10.1111/j.1460-9568.2012.08175.x

18. Tecchio F, Zappasodi F, Assenza G, Tombini M, Vollaro S, Barbati G, et al. Anodal transcranial direct current stimulation enhances procedural consolidation. J Neurophysiol. 2010; 104: 1134–1140. doi:10.1152/jn.00661.2009

19. Hummel FC, Heise K, Celnik P, Floel A, Gerloff C, Cohen LG. Facilitating skilled right hand motor function in older subjects by anodal polarization over the left primary motor cortex. Neurobiol Aging. 2010; 31: 2160–2168. doi:10.1016/j.neurobiolaging.2008.12.008

20.   Nitsche MA, Jaussi W, Liebetanz D, Lang N, Tergau F, Paulus W. Consolidation of human motor cortical neuroplasticity by D-cycloserine. Neuropsychopharmacology. 2004; 29: 1573–1578. doi:10.1038/sj.npp.1300517

21.   Fritsch B, Reis J, Martinowich K, Schambra HM, Ji Y, Cohen LG, et al. Direct current stimulation promotes BDNF-dependent synaptic plasticity: Potential implications for motor learning. Neuron . 2011; 66: 198–204. doi:10.1016/j.neuron.2010.03.035.

22.   Nitsche MA, Seeber A, Frommann K, Klein CC, Rochford C, Nitsche MS, et al. Modulating parameters of excitability during and after transcranial direct current stimulation of the human motor cortex. J Physiol. 2005; 568: 291–303. doi:10.1113/jphysiol.2005.092429

23.   Floyer-Lea A, Wylezinska M, Kincses T, Matthews PM. Rapid modulation of GABA concentration in human sensorimotor cortex during motor learning. J Neurophysiol. 2006; 95: 1639–1644. doi:10.1152/jn.00346.2005

24.   Stagg CJ, Bachtiar V, Johansen-Berg H. The role of GABA in human motor learning. Curr Biol. 2011; 21: 480–484. doi:10.1016/j.cub.2011.01.069

25.   Ziemann U, Hallett M, Cohen LG. Mechanisms of deafferentation-induced plasticity in human motor cortex. J Neurosci. 1998; 18: 7000–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/9712668

26.   Classen J, Liepert J, Wise SP, Hallett M, Cohen LG. Rapid plasticity of human cortical movement representation induced by practice. J Neurophysiol. 1998; 79: 1117–1123. doi:citeulike-article-id:2143137

27.   Rosenkranz K, Kacar A, Rothwell JC. Differential modulation of motor cortical plasticity and excitability in early and late phases of human motor learning. J Neurosci. 2007; 27: 12058–12066. doi:10.1523/JNEUROSCI.2663-07.2007

28.   Pascual-Leone a, Grafman J, Hallett M. Modulation of cortical motor output maps during development of implicit and explicit knowledge. Science. 1994; 263: 1287–1289.

29.   Bütefisch CM, Davis BC, Wise SP, Sawaki L, Kopylev L, Classen J, et al. Mechanisms of use-dependent plasticity in the human motor cortex. Proc Natl Acad Sci U S A. 2000; 97: 3661–5. doi:10.1073/pnas.050350297

30. Rosenkranz K, Williamon A, Rothwell JC. Motorcortical excitability and synaptic plasticity is enhanced in professional musicians. J Neurosci. 2007; 27: 5200–5206. doi:10.1523/JNEUROSCI.0836-07.2007

31. Zhang X, de Beukelaar TT, Possel J, Olaerts M, Swinnen SP, Woolley DG, et al. Movement observation improves early consolidation of motor memory. J Neurosci. 2011; 31: 11515–11520. doi:10.1523/JNEUROSCI.6759-10.2011

32. Muellbacher W, Ziemann U, Wissel J, Dang N, Kofler M, Facchini S, et al. Early consolidation in human primary motor cortex. Nature. 2002; 415: 640–644. doi:10.1038/nature712

33. Bisio A, Avanzino L, Gueugneau N, Pozzo T, Ruggeri P, Bove M. Observing and perceiving : a combined approach to induce plasticity in human motor cortex. Clin Neurophysiol. 2014; . doi:10.1016/j.clinph.2014.08.024

34. Schwartz AB. Useful signals from motor cortex. J Physiol. 2007 Mar 15; 579 (Pt 3):581-601. Epub 2007 Jan 25

35. Wiestler T, Diedrichsen J. Skill learning strengthens cortical representations of motor sequences. Elife. 2013; 2013: 1–20. doi:10.7554/eLife.00801

36. Missitzi J, Gentner R, Misitzi A, Geladas N, Politis P, Klissouras V, et al. Heritability of motor control and motor learning. Physiol Rep. 2013; 1: e00188. doi:10.1002/phy2.188

37. Oldfield RC. The assessment and analysis of handedness: the Edinburgh inventory. Neuropsychologia. 1971; 9: 97–113. doi:10.1016/0028-3932(71)90067-4

38. Rickham PP. Human Experimentation. Code of Ethics of the World Medical Association. Declaration of Helsinki. Br Med J. 1964; 2: 177.

39. Alaerts K, Swinnen SP, Wenderoth N. Is the human primary motor cortex activated by muscular or direction-dependent features of observed movements? Cortex. ; 2009; 45: 1148–1155. doi:10.1016/j.cortex.2008.10.005

40. Reis J, Schambra HM, Cohen LG, Buch ER, Fritsch B, Zarahn E, et al. Noninvasive cortical stimulation enhances motor skill acquisition over multiple days through an effect on consolidation. Proc Natl Acad Sci U S A. 2009; 106: 1590–1595. doi:10.1073/pnas.0805413106

41.   Reis J, Fischer JT, Prichard G, Weiller C, Cohen LG, Fritsch B. Time- but Not Sleep-Dependent Consolidation of tDCS-Enhanced Visuomotor Skills. Cereb Cortex. 2013; 1–9. doi:10.1093/cercor/bht208

42.   Galea JM, Vazquez A, Pasricha N, Orban De Xivry JJ, Celnik P. Dissociating the roles of the cerebellum and motor cortex during adaptive learning: The motor cortex retains what the cerebellum learns. Cereb Cortex. 2011; 21: 1761–1770. doi:10.1093/cercor/bhq246

43.   Saucedo Marquez CM, Zhang X, Swinnen SP, Meesen R, Wenderoth N. Task-specific effect of transcranial direct current stimulation on motor learning. Front Hum Neurosci. 2013; 7: 333. doi:10.3389/fnhum.2013.00333

44.   Javadi AH, Cheng P. Transcranial direct current stimulation (tDCS) enhances reconsolidation of long-term memory. Brain Stimul. 2013; 6: 668–674. doi:10.1016/j.brs.2012.10.007

45.   Soekadar SR, Witkowski M, Birbaumer N, Cohen LG. Enhancing Hebbian Learning to Control Brain Oscillatory Activity. Cereb Cortex. 2014; 1–7. doi:10.1093/cercor/bhu043

46.   Parikh PJ, Cole KJ. Effects of transcranial direct current stimulation in combination with motor practice on dexterous grasping and manipulation in healthy older adults. Physiol Rep. 2014; 2: e00255. doi:10.1002/phy2.255

47.   Peters MA, Thompson B, Merabet LB, Wu AD, Shams L. Anodal tDCS to V1 blocks visual perceptual learning consolidation. Neuropsychologia. 2013; 51: 1234–1239. doi:10.1016/j.neuropsychologia.2013.03.013

48.   Monte-Silva K, Kuo MF, Hessenthaler S, Fresnoza S, Liebetanz D, Paulus W, et al. Induction of late LTP-like plasticity in the human motor cortex by repeated non-invasive brain stimulation. Brain Stimul. 2013; 6: 424–432. doi:10.1016/j.brs.2012.04.011

49.   Bortoletto M, Pellicciari MC, Rodella C, Miniussi C. The Interaction With Task-induced Activity is More Important Than Polarization : A tDCS Study. Brain Stimul. 2015; 8: 269–276. doi:10.1016/j.brs.2014.11.006

50.   Müller-Dahlhaus F, Ziemann U. Metaplasticity in Human Cortex. Neuroscientist. 2014; doi:10.1177/1073858414526645

51.   Alonzo A, Brassil J, Taylor JL, Martin D, Loo CK. Daily transcranial direct current stimulation (tDCS) leads to greater increases in cortical excitability than second daily transcranial direct current stimulation. Brain Stimul. 2012; 5: 208–213. doi:10.1016/j.brs.2011.04.006

52.   Meinzer M, Jähnigen S, Copland DA, Darkow R, Grittner U, Avirame K, et al. Transcranial direct current stimulation over multiple days improves learning and maintenance of a novel vocabulary. Cortex. 2013; 50. doi:10.1016/j.cortex.2013.07.013

53.   Furuya S, Klaus M, Nitsche MA, Paulus W, Altenmuller E. Ceiling Effects Prevent Further Improvement of Transcranial Stimulation in Skilled Musicians. J Neurosci 2014; 34: 13834–13839. doi:10.1523/JNEUROSCI.1170-14.2014

54.   Convento S, Bolognini N, Fusaro M, Lollo F, Vallar G. Neuromodulation of parietal and motor activity affects motor planning and execution. Cortex.   2014; 57: 51–59. doi:10.1016/j.cortex.2014.03.006

Chapter four

The effect of tDCS on motor recovery in an animal model of stroke

Orjon Rroji, Daniel Woolley, Kris van Kuyck, Marie-Eve Lamamée, Lutgarde Arckens, Britta Fritsch, Bart Nuttin, Nicole Wenderoth.

ABSTRACT

Previous research in stroke patients and stroke animal models has suggested that tDCS might be a suitable tool to promote functional recovery early after stroke. tDCS can augment synaptic plasticity in vitro and in vivo with effects being larger when it is applied together with synaptic activity (which might result from motor training) leading to enhanced LTP-like plasticity. However, it is currently unclear whether tDCS early after stroke is truly beneficial for functional recovery in humans and which mechanisms might rive this effect. This gap in knowledge results partly from the lack of animal models that are translatable to clinical applications. Here we applied anodal versus sham tDCS over ipsilesional primary cortex (M1) in a rat stroke model while animals were trained on the pasta matrix reaching task. We induced photothrombotic stroke in M1 after 10 days of grasping training. Starting from the 4th day post-infarct the animals were trained for 10 more days while anodal tDCS (15.9 A/m^2 current density) or sham tDCS was concurrently applied for 30 min per day. Rats with large bilateral lesions did hardly grasp after surgery. After removing these rats from the analysis, the anodal tDCS group recovered grasping function significantly better ($p < 0.001$) compared to sham tDCS group. Our main finding is that applying tDCS to the ipsilesional cortex during 30 min of motor training in a rat stroke model is feasible. Moreover, combining motor training with anodal tDCS might benefit recovery when compared to training with sham tDCS.

INTRODUCTION

Stroke is the most common cause for acquired adult disability in developing countries. Immediately after the event, patients commonly suffer from severe to moderate motor impairment of the lower and upper limbs. Fortunately, most patients undergo some form of "functional recovery", and goal directed behaviour is often at least partly restored. Note however that functional recovery, particularly of the upper limb, is rarely complete and approximately 80% of stroke patients suffer from permanent arm paresis that can vary from a mild impairment to being highly disabling (Kolominsky-Rabas et al., 2001).

During the last years, research in animal models has shown that functional recovery results either from true recovery/repair of neurons located in the peri-infarct zone or from reorganization which often happens at the system's level to compensate for lost function (Murphy & Corbett, 2009). Both processes depend on the brain's ability to undergo neuroplastic changes including synaptic strengthening and activity-dependent rewiring of neurons. Growing evidence from animal models suggests that there is a sensitive period of enhanced post-ischemic plasticity early after the incident caused by a net increase in the expression of growth promoting versus growth inhibiting genes within stroke affected circuits, thus providing a supportive milieu for neuroplastic changes to occur (Murphy & Corbett, 2009). Rehabilitation training positively interacts with this plasticity enhancing environment (Zeiler and Krakauer, 2012) and even though it is unclear when the post-ischemic sensitive period starts or how long it lasts there is evidence that early rehabilitation training (commencing 5-14 days after the insult in rats) results in more efficient recovery than training starting later after the insult (e.g. commencing 30 days post-stroke) (Biernaskie et al., 2004).

One important question is whether functional recovery early after stroke can be further augmented by new interventions, one of them being transcranial Direct Current Stimulation (tDCS). tDCS is a well-tolerated technique that has been applied for many years in human volunteers. The basic mechanism is that tDCS causes subthreshold modulation of neuronal membrane potentials, which alters cortical activity depending on the current flow direction through the tissue (Nitsche & Paulus, 2000). When anodal tDCS is applied to human motor cortex (M1) at rest it increases corticomotor excitability with after-effects outlasting the stimulation period (typically 13-30 min) by up to 120 minutes (Nitsche & Paulus, 2001). Furthermore, parallel experiments in animal models and humans have shown that anodal tDCS can augment synaptic plasticity in vitro and in vivo. One important finding is that effects are largest when tDCS is applied *together* with synaptic activation (caused either by voluntary motor activity or, when tested in vitro, by plasticity inducing stimulation protocols) leading to enhanced LTP-like plasticity (Fritsch et al., 2011; Stagg et al., 2011). Even though

the underlying mechanism is not completely understood, it has been suggested that tDCS facilitates activity-dependent BDNF secretion (Fritsch et al., 2011).

Anodal tDCS applied to the ipsilesional cortex during training has demonstrated potential to modulate neuroplasticity and motor learning with the upper limb in chronic stroke patients (Fregni et al., 2005; Hummel & Cohen, 2006; Hummel et al., 2005; Lefebvre et al., 2012). By contrast, much less is known about its efficacy when applied during the (sub)acute phase of stroke recovery, i.e. early after the incident. The few studies that investigated the application of tDCS in (sub)acute stroke patients revealed mixed effects on functional recovery of the arm or hand when applying anodal tDCS to ipsilesional M1. Rossi et al, (2013) found no effect on recovery when tDCS was applied while acute stroke patients were at rest. Others applied tDCS together with different forms of rehabilitation training in subacute patients (Hesse et al., 2007; Kim et al., 2009) but reported no significant effect on functional outcomes, whereas Sattler et al., (2015) combined anodal tDCS over the ipsilesional motor cortex with repetitive peripheral nerve stimulation during the first 4 weeks post stroke and reported positive effects on functional recovery. These studies show that it is principally feasible to apply anodal tDCS in (sub)acute stroke patients but it is not clear whether anodal tDCS in combination with motor training is an appropriate tool to facilitate early functional recovery of the upper limb.

Currently, the design of appropriate clinical trials in humans is hampered because more mechanistic insights into cellular and molecular mechanisms through which tDCS might facilitate post stroke recovery are lacking. Animal models of stroke could fill this gap of knowledge and previous studies have tested the effect of tDCS in rodent models (Table 1) to investigate either the protective effect of cathodal tDCS applied to the ipsilesional hemisphere within the first minutes to hours after stroke, i.e. during the acute phase (Notturno et al., 2014; Peruzzotti-Jametti et al., 2013) or they investigated the plasticity-enhancing influence of anodal tDCS applied to the ipsilesional hemisphere for several days during the subacute phase (at least 1 day after the incident) (Jiang et al., 2012; Kim et al., 2010; Yoon et al., 2012).

However, studies investigating the plasticity-enhancing effect of ipsilesional anodal tDCS used poorly-translatable stimulation protocols because tDCS was applied while rats were anesthetized (Kim et al., 2010; Yoon et al., 2012) or at least at rest (Jiang et al., 2012). Note however, that from the methods description in Jiang et al., (2012) it is not clear whether they applied a constant current or changed between stimulation and no stimulation every 100 ms. The reported effects of anodal tDCS on motor recovery are inconsistent across studies. Yoon et al., (2012) showed that 5 days of tDCS versus sham stimulation facilitated recovery of gross motor function as measured by the beam balance test and the motor behavioural index with rats that started receiving stimulation 1 week post stroke faring better than those that started receiving stimulation 1 day post stroke (Yoon et al., 2012). Jiang et al.,

(2012) report a potential trend for better recovery in the tDCS compared to the sham group when tDCS is applied for 7 days or longer. However, in this study performance was measured only at one single time point, making their data difficult to interpret because the experimentally induced stroke can affect function in a highly variable manner. Kim at al., (2010) found no significant effect of anodal tDCS on motor function when applied early after stroke, however they used an unusually large stimulation electrode for rats (1 cm diameter). Additionally, all three previous studies report some beneficial effects of tDCS on neural parameters. Kim et al., (2010) reported that anodal tDCS had no effect on infarct size, but white matter axons were better preserved in rats that received anodal tDCS. Yoon et al., (2012) showed that anodal tDCS starting 1 week post stroke promotes Gap-43 expression in intact cortex. Gap-43 has been shown to play an important role in axonal growth and the formation of new connections (Benowitz & Routtenberg, 1997). Finally, Jiang et al., (2012) reported an increase in dendritic spine density in the anodal tDCS group, suggesting that tDCS might play a role in promoting neural plasticity.

In summary, previous studies have tested the effects of applying anodal or cathodal tDCS during the acute phase of the stroke, i.e. a few minutes or hours after infarction (Notturno et al., 2014; Peruzzotti-Jametti et al., 2013) or during the subacute phase, i.e. up to 2 weeks after the induction of experimental stroke. The latter studies could provide a model for applying tDCS during stroke rehabilitation in humans, however, they have a major limitation that tDCS was applied at rest or under anaesthesia (Jiang et al., 2012; Kim et al., 2010; Yoon et al., 2012) and, therefore, these studies do not provide insights as to whether anodal tDCS would boost neuroplastic changes triggered by rehabilitation training.

The purpose of the present study was to develop an animal model of stroke rehabilitation that better mimics tDCS applications in humans using a bedside-to-bench approach. Establishing such an animal model would open new opportunities to answer practical questions, for example, to determine a dose-response relationship for applying tDCS in stroke, as well as to gain more mechanistic insights into how tDCS affects recovery which, in turn, might be used to optimize tDCS treatment in humans.

For these reasons, we aimed to develop an animal model where the effect of anodal tDCS over ipsilesional M1 is tested while animals perform goal-directed upper limb training which mimics rehabilitation training in patients. Therefore, rats were trained on the pasta matrix reaching task, a sensitive behavioural assay that allows the manipulation of limb use in order to mimic human clinical phenomenon (Kerr & Tennant, 2014). After 10 days of training on the pasta matrix reaching task we induced a photothrombotic stroke in the primary motor cortex contralateral to the preferred reaching and grasping limb. The photothrombotic stroke model in rat uses local intravascular photo-oxidation to generate a highly circumscribed ischemic cortical lesion (Watson et al, 1985). Several studies provide evidence that the photothrombotic occlusion of cerebral microvessels using a

photosensitive dye (Rose Bengal) is an effective and minimally invasive method of simulating human stroke conditions (Dietrich et al, 1986; Labat-gest & Tomasi, 2013; Moon et al., 2009; Pevsner et al., 2001; Schmidt et al., 2012; Watson et al., 1985). Rats were then trained on the pasta matrix reaching task for 10 days post stroke (starting at day 4 after the infarct) with the concurrent application of either anodal tDCS or sham tDCS. In this randomized, double blind sham controlled study we hypothesized that animals receiving anodal tDCS improve more in reaching and grasping performance than those that receive sham tDCS.

The effect of tDCS on motor recovery in an animal model of stroke

Study	Species	Stroke induced	tDCS influences recovery	tDCS polarity	tDCS state (rest or with task)	Time post-stroke for first tDCS application (min/h/d)	Time (min & days)	Current density (A/m^2)	Intensity (µA)	Motor performance measured	Electrode size
(Jiang et al., 2012)	Rat	MCAO	Yes	Anode & cathode	N.R.	1d	30min, daily, for 3, 7, 14d	n.r.	100	BWT	Active=10mm diameter, Reference=3x3cm^2
(Yoon et al., 2012)	Rat	MCAO	Yes	Anode	General anesthesia	1d, 7d	20min, daily for 5d	28.2	200	BB, MBI, RR, BM	Active=Cup shaped, Cathodal=80x60mm
(Kim et al., 2010)	Rat	MCAO	Yes	Anode & cathode	General anesthesia	2d	30min, daily for 2 weeks	1.27	100	Garcia test, RR, mFFT	Active=10mm diameter, Reference=3x3cm^2
(Peruzzotti-Jametti et al., 2013)	Mouse	pMCAO	Yes	Anode & cathode	General anesthesia	30 min or/and 4.5h	20min	55	250	No	Active=2.4mm diameter, Reference=5.2cm^2
(Notturno et al., 2014)	Rat	MCAO	Yes	Cathode	General anesthesia	0 min & 45 min	15min	28.6	200	No	Active=3mm diameter, Reference=10.5cm^2
Present study (2015)	Rat	PhT	Yes	Anode	Training	4d	30 min, daily for 10 d	15.9	50	PMRT	Active=2mm diameter, Reference=6.25cm^2

MCAO = Middle Cerebral Artery Occlusion; **pMCAO** = proximal Middle Cerebral Artery Occlusion; **PhT** = PhotoThrombotic; **BWT** = Beam Walking Test; **BB** = Beam balance; **MBI** = Motor behaviour index; **RR** = Rotarod; **BM** = Barnes maze; **Garcia test** = Motor behaviour test; **mFFT** = modified Foot Fault Test; **PMRT** = Pasta Matrix Reaching Task; **N.R.** = Not Reported

Table 1: Summary of studies, including the experiment reported here, which have tested   the effects of tDCS on recovery in rodent models of stroke.

MATERIALS AND METHODS

Subjects

The experiment was performed on 28 male Sprague – Dawley rats (Harlan Laboratories B.V., The Netherlands) with a mean body weight of 280 ± 15 g at the start of the experiment. They arrived two weeks prior to testing in order to familiarize with the new environment. The animals were kept in Individually Ventilated Cages (IVC) standard single cages under laboratory conditions of 14 h light / 10 h dark (lights on at 7:00 A.M. and off at 9:00 P.M.). The room temperature was kept constant at 22 °C. The animals were on a daily food restriction schedule (a mix of standard chow and Capellini pasta of ± 10 g per day) that reduced their body weight to 90% in order to keep rats motivated to reach for food. Water was provided *ad libitum*. The experiment was performed during the day in the light phase. This research project and the experimental protocol were approved by the KU Leuven ethics committee for laboratory experimentation (project number: P104/2013), and was in accordance with the Belgian and European laws, guidelines and policies for animal experimentation, housing and care (Belgian Royal Decree of 29 May 2013 and European Directive 2010/63/EU on the protection of animals use for scientific purposes of 20 October 2010).

Of the 28 rats that started the experiment, five did not show sufficient improvement in grasping performance during pre-tDCS training (i.e. performance did not reach a plateau), two died during surgery and four had an abnormal lesion size or location. Of these four rats with abnormal lesions, two rats were excluded right after the extraction of the brain because in one rat the lesion was visibly too small and in the other rat the lesion was far away from the target area, i.e. primary motor cortex area. Two more rats (rat 9 and rat 14, table 2) were excluded after the histology was performed because the lesion size (lateral-medial diameter x dorsal caudal diameter) of the damaged hemisphere (i.e. ipsilesional) deviated from the group mean by more than 1.5 standard deviations (average lesion size was 2.6 mm in lateral-medial axis and 1.4 mm along in dorsal-caudal axis). Table 2 shows the lesion size of every animal including the two animals (indicated in bold) that were excluded based on their lesion size derived from histology. The total number of animals included in the final set of analyses was n = 17 of which 9 rats had a histological examination (see further details below).

| Rat | Damaged (ipsilesional) hemisphere | | Other (contralesional) hemisphere | |
	Lateral-medial (mm)	Dorsal-caudal (mm)	Lateral-medial (mm)	Dorsal-caudal (mm)
Rat 1	3.1468	1.0499	2.2708	1.0903
Rat 3	2.2074	0.9386		
Rat 6	3.0633	1.8077	2.8444	1.4442
Rat 8	2.4872	1.4949	0.5177	0.3109
Rat 9	3.5614	2.0921	0.8494	0.3038
Rat 10	2.4132	0.9256	0.8353	0.4959
Rat 11	2.5134	1.5797		
Rat 13	3.0269	1.7935	1.982	1.1138
Rat 14	1.5719	0.689		
Rat 15	2.9534	1.9746		
Rat 16	3.1264	1.8491	1.1278	0.623

Table 2: Lesion size, showed in mm, of each animal (n = 11) in the target (damaged) hemisphere and the non-target (other) hemisphere. Rat 9 and Rat 14 (printed in bold) where excluded because the lesion size in the damaged (i.e. ipsilesional) hemisphere differed from the group mean by more than 1.5 standard deviations.

Motor task: pasta matrix reaching and grasping task

The test apparatus for the pasta matrix motor task was similar to that used previously (Ballermann et al., 2001; Ballermann et al., 2000). The task allows for quantitative (number of successful reaches/ transports) assessment of behavioural performance and provides a sensitive measure of both motor impairment and improvement after stroke. During testing a single rat was placed into a transparent plastic box (35 cm long x 35 cm high x 14 cm wide). A 1 cm wide slot was located at the front of the box through which the animal could reach and grasp the pasta. Adjacent to the slot was a matrix 6 cm wide, 4 cm high and 12 cm wide. The matrix contained an array of holes (12 rows deep by 20 rows wide). Holes had a diameter of 2 mm and centre-to-centre distance of 4 mm. The pasta pieces (Capellini pasta, approximately 1.3 mm in diameter) were inserted vertically into the holes of the matrix and extended by 2.5 cm. During habituation both sides of matrix were filled (240 pieces of pasta, Fig 1) so that animals could grasp with either limb. The limb that was used most frequently for grasping during the habituation phase was defined as the preferred limb. During pre-surgery and post-surgery training only one side of the matrix was filled enforcing grasping with the preferred limb only. For example, when only the left side of the matrix was filled, the animal was forced to use the right limb to reach and grasp. A high-speed camera (Sony HDR-AS15, frame rate 30 per second,

resolution 1080p) was used to record each behavioural session for offline analyses (see data analysis for the measures used to assess performance).
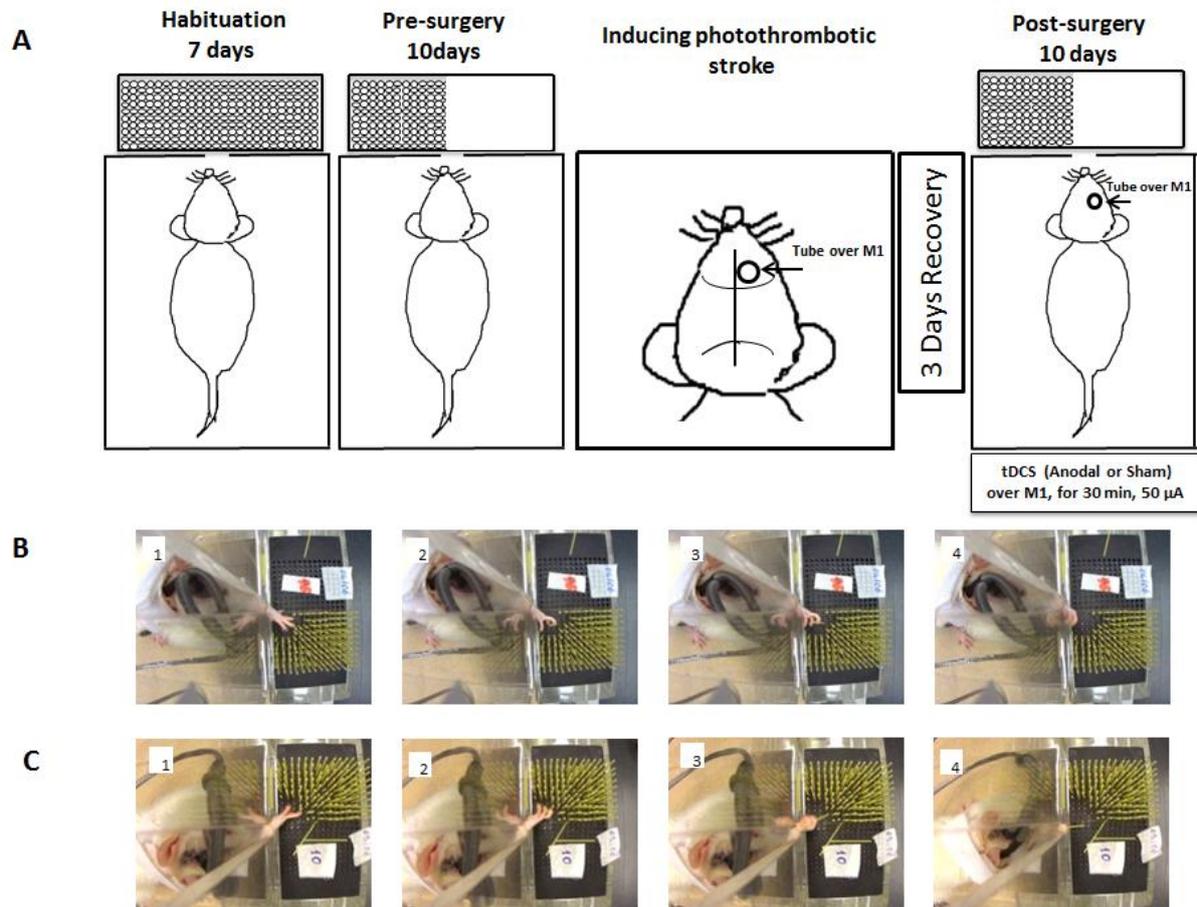

Figure 1: **Experimental procedures.** A: Pasta matrix reaching task device (schematic overview). The matrix is filled with 240 pieces of pasta (habituation phase) or 120 pieces of pasta (pre and post-surgery training). Photothrombotic stroke was induced and the tube was mounted over M1. Rats had three days of recovery before starting the behavioural training while 50 μA for 30 min was delivered over M1. **B:** The animal is able to grasp (2) and break (3) the pasta but could not transport it through the gap into the testing box. This attempt was scored as successful grasp (G_s) **C:** The animal is able to grasp (2), break (3) and transport (4) the pasta into the testing box. This attempt was scored as a successful grasp and transport ($G\&T_s$).

Photothrombotic stroke and epicranial tube placement

Each animal was anesthetized via intraperitoneal administration of an anaesthetic mixture of 0.6 mL Ketamine (Nimatek) + 0.4 mL Medetomidine (Narcostart) + 1 mL sodium chloride (NaCl) 150 µl / 100 g body weight. The depth of anaesthesia was verified by foot pinches. An adequately anesthetised rat should not give a foot response such as flexion or withdrawal. Using an electric razor the scalp and thigh of the rat were shaved. The animal was fixed in a stereotactic frame which allowed us to define the coordinates of the motor cortex in relation to bregma. Body temperature was maintained constant during the whole surgical procedure at 37°C using a thermostatically controlled heating pad. The scalp was longitudinally incised for 2.5 cm and retracted to expose the skull. The periosteum was gently removed until coronal and sagittal sutures were visible. Cotton swabs were used to clean the skull. The femoral vein was exposed and cannulated with a catheter 24G x ¾" (0.7 x 19 mm) and Rose Bengal was injected at 22 ml/min (Braun, Melsungen, Germany). We induced photothrombotic stroke via a cold light source with an aperture of 8 mm diameter (150 W, KL 1500 LCD, 3000K – Olympus Company) stereotactically positioned onto the skull after skin incision. The coordinates were: 1.5 mm anterior to bregma and 2.5 mm lateral to midline. In order to trigger a photochemical reaction in the system we administered Rose Bengal (1.3 ml/kg) intravenously via the femoral vein through the previously inserted catheter, 2 minutes before illumination. Rose Bengal leads to local blood clotting when activated by light. The light locally produces single oxygen (free radicals) when reacting with Rose Bengal, which in turn peroxidizes lipid molecules in the vascular endothelium, causing platelets and erythrocytes to aggregate and thus leading to an occlusion of small vessels (Dietrich et al., 1986; Watson et al., 1985). For each rat, stroke was induced in the hemisphere contralateral to the preferred limb. After induction of the lesion, we attached a tube (outer diameter 3.86 mm, inner diameter 2 mm, custom made) onto the skull (1.5 mm anterior to bregma and 2.5 mm lateral from midline) for providing tDCS during post-surgery training. To firmly attach the tube we fixed metal screws (Fine Science Tools, item-no 19010-00) to the skull in close proximity of the tube and sealed the surrounding skull, screws and tube with dental cement (Tetric Evo Flow, Ivoclar Vivadent AG, Schaan, Liechtenstein). The aim of applying dental cement was to hold the tube in place. The scalp and thigh wound were stitched and a pain killer (0.2 mL of Metacam + 0.8 mL of NaCl) 100 µl/100 gr body weight was injected subcutaneously. After the surgical procedures, the rats were returned to their home cage and placed over a heating pad. The body temperature was controlled and kept constant at 37 °C for the first night post-surgery. Three days of recovery preceded the start of post-surgery training.

Transcranial Direct Current Stimulation (tDCS) setup

A constant current stimulator (A-M systems, Inc. isolated Pulse Stimulator Model 2100 WA 98324, U.S.A.) was used to deliver tDCS over the ipsilesional hemisphere during the post-surgery motor training. tDCS was applied by inserting the anode (round, 2 mm diameter copper alloy electrode, custom made) into the tube fixed to the skull and using NaCl solution as the conductive medium (Liebetanz et al., 2006). The reference electrode (cathode) was a large (2.5 cm x 2.5 cm) conductive carbon rubber electrode (Enraf Nonius B.V Delft, The Nederlands) that was placed onto the ventral thorax of the unrestrained animal using a rat jacket (Rat jacket med 62-0058, Harvard Apparatus, France). In accordance with previous research (Liebetanz et al., 2006; Rueger et al., 2012; Wachter et al., 2011), a constant current of 0.05 mA (corresponding to a current density of 15.9 A/m^2) was delivered through the anodal electrode for 30 min duration in each training session. Stimulation at this intensity and duration does not cause discomfort, pain or damage to neural tissue (Liebetanz et al., 2009) while it was shown to effectively modulate brain activity (Jiang et al., 2012; Kim et al., 2010; Notturno et al., 2014; Yoon et al., 2012). At the start of the training session, anodal tDCS was applied by manually ramping up current intensity over 5 s to achieve conditions similar to human tDCS application. Once the intensity reached 0.05 mA it was kept constant for 30 min. We continuously monitored the device to ensure that stimulation was being applied. After 30 min it was manually ramped down over the same time period. For sham tDCS, montage of the electrode as well as the procedure of ramping the current up and down were identical but no current was delivered through the electrodes.

Experimental protocol

Our overall experimental protocol consisted of (I) habituation, (II) pre-surgery training, (III) surgery (inducing photothrombotic stroke and mounting the tube for tDCS delivery), (IV) recovery, and (V) post-surgery training (Fig 1).

Habituation was performed over 7 consecutive days. During habituation the animals were first familiarised with the apparatus and learned to grasp pieces of pasta for 30 min each day. In order to obtain reliable data during testing, reducing stress is fundamental (Schallert et al., 2000). At the start of habituation the animals could use both limbs to reach to either the left or right side of the pasta matrix. They did not wear the jacket and no electrode cables or other equipment was present in the box. Two days after they started grasping they were habituated to wearing the jacket. When the animals were familiarised with the jacket and reached and grasped for pasta for two days, they were

habituated to wearing the jacket with the reference electrode inserted and wired to a swivel at the top of the cage. Video analysis was performed to check which limb was most frequently used to grasp the pieces of pasta to determine the preferred limb for each rat individually.

Pre-surgery training was performed for 10 consecutive days. Training started every day at the same time and in the same room, with the light and room temperature constant for the duration of the experiment. Animals were forced to use their preferred limb by filling only one half of the pasta matrix (i.e. opposite to the preferred limb). Pilot research in our lab indicated that a healthy and hungry rat tends to grasp all possible reachable pieces of pasta within 10 minutes. We provided the rats with 15 min, i.e. enough time to grasp as much pasta as possible. After 15 minutes, the matrix was refilled and rats had another 15 minutes to grasp as much as possible. In total, 30 minutes of grasping training was performed each day. After 10 days of pre-surgery training, the surgery was performed and the hemisphere controlling the preferred limb was lesioned as described above.

After surgery, all rats were given 3 days to recover. Therefore, the animals were returned to their home cage and checked daily for infections. Animals were given *ad libitum* access to food on the day immediately following surgery. Starting from the second day post-surgery the amount of food was again decreased in order to keep the animals at a body weight similar to pre-surgery. There was no significant difference between pre and post-surgery $F_{(9, 270)} = 1.27$, $p > 0.05$ body weight. No infection or anomalies were encountered.

Post-surgery training was performed for 10 consecutive days. Rats were randomly assigned to either the anodal tDCS or sham tDCS group with the experimenter being blinded to the stimulation condition during post-training and video scoring. Similar to pre-surgery training, post-surgery training was performed once per day for 30 min (2 x 15 min). Animals were again forced to use their paretic limb (contralateral to the lesioned hemisphere) and stimulation was applied concurrently with training.

Histology

At the completion of the experiment the animals were euthanized with an overdose of sodium pentobarbital (3 ml Nembutal, Ceva Sante Animale, Brussels, Belgium) 1 ml/100 g body weight. They were then intracardially perfused with sucrose (100 gr sucrose in 1000 ml of distilled H_2O) followed by 4% paraformaldehyde (Acros Organics, Geel, Belgium). Brains were carefully removed and kept in 4% paraformaldehyde solution for 4-5 days, then put through a dehydrating series of graded concentrations of alcohol, and finally embedded in paraffin. A microtome device (Microm cool-cut HM 360 Prosan, Germany) was used to make 10 μm-thick coronal sections. Sections were

deparaffinised in xylol, followed by rehydratation in a graded alcohol series in distilled water. Deparaffinised sections were Nissl-stained in a filtered 1% cresyl-violet acetate solution (Sigma C-5042, 10 g dissolved in 1 L of distilled H_2O), dehydrated in a graded alcohol series, cleared with xylene and cover-slipped with DePex, to determine the layers of the rat neocortex and the size and position of the cortical lesions (Fig 2). Hereto, the stained coronal sections were analysed and imaged under a microscope (Zeiss Axio imager Z1) equipped with an AxioCam MRm camera, using the software program Zen Pro 2012 (Carl Zeiss, Benelux). Each section/picture was compared with a stereotactic rat brain atlas (Paxinos & Watson, 2007), to record the exact position of the lesion along the anterior-posterior and medio-lateral axis of the brain. Digital images of sections were also analysed using Image J (www.imagej.nih.gov) to quantify lesion size. Length, width and depth were measured and the overall lesion volume within each hemisphere was approximated by a half ellipsoid (4/3*pi*length*depth*width)/2. The experimenter remained blinded during lesion quantification.

Unfortunately 8 brains were not useable for histology due to a technical problem during slicing. We analysed the remaining 9 data sets, 3 from sham tDCS rats and 6 from anodal tDCS rats.
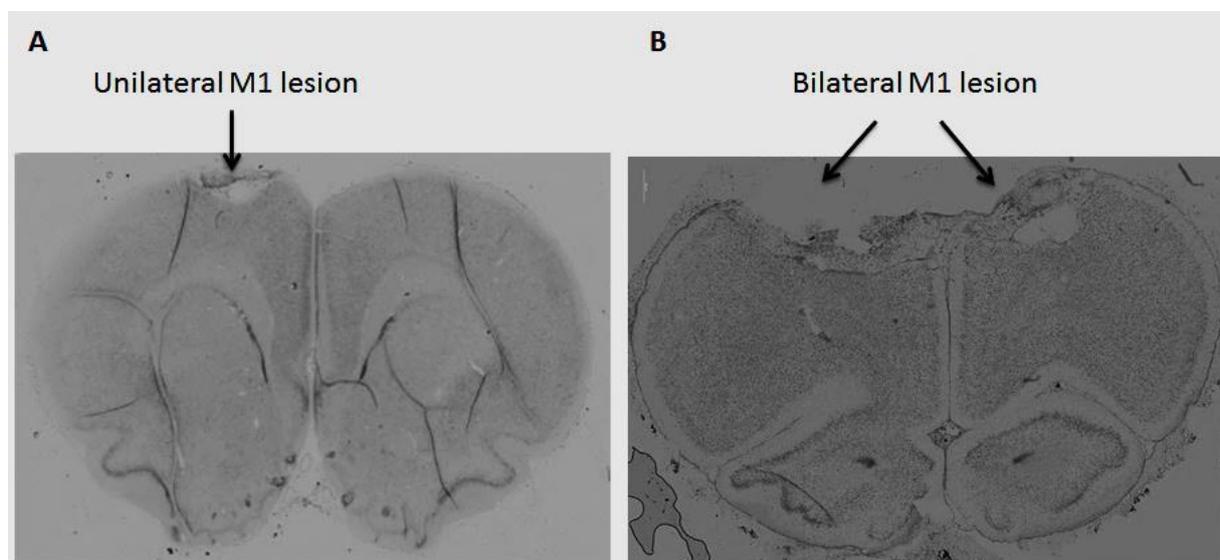

Figure 2: Histology of the photothrombotic stroke lesion. Deparaffinised sections were Nissl-stained in a filtered 1% cresyl violet. 10 μm-thick coronal sections were made for each rat brain. A – Unilateral lesion corresponds at bregma 3 mm. B – bilateral lesion size corresponds at bregma 4.20 mm. Bar scale (1000 μm) on the top of the B figure.

Data analyses

Offline analyses were performed based on the videos recorded during the pre–surgery and post–surgery training sessions. The experimenter scoring the videos was blind as to whether rats received anodal tDCS or sham tDCS. We determined the following parameters:

1. Number of successful grasps (G_s): the animals were able to grasp and break the pasta, however, they could not transport it through the gap into the testing box (Fig 1B).

2. Number of successful grasp and transports ($G\&T_s$): the animals were able to grasp, break and transport the pasta into the testing box (Fig 1C).

3. Grasps with non-paretic limb ($G_{healthy}$): even though the pasta was positioned so that it was ideally reached with the paretic limb, the animals occasionally grasped with the non-paretic limb.

Statistics

We first analysed performance (G_s and $G\&T_s$) over the course of pre-surgery training to determine whether the sham tDCS and the anodal tDCS groups exhibited similar learning curves. All variables were subjected to repeated analysis of variance (ANOVA) with the factors *Time* (pre1-pre10) and *Group* (anodal tDCS vs sham tDCS).

Next we tested whether grasping performance was impaired by the experimental lesion. Since there was large session-to-session variability in the grasping parameters of the pre-surgery training and to be consistent with the approach we used to calculate maximal training gains (see below), we estimated grasping proficiency via the maximum performance score reached prior to the surgery (maxPRE). A *Time* x *Group* repeated measures ANOVA and appropriate post-hoc tests were calculated to compare maxPRE versus post-4 performance (first day of post-surgery training) for both groups.

Recovery was quantified relative to pre-surgery performance by normalizing all post-surgery performance data to maxPRE: G_s post$_i$ = G_s post$_i$/ G_s maxPRE; $G\&T_s$ post$_i$ = $G\&T_s$ post$_i$/ $G\&T_s$ maxPRE with i ε [4..13]. Normalized successful grasp and successful grasp & transport data were subjected to a repeated measures ANOVA with the factors *Time* (post4-post13) and *Group* (anodal tDCS vs sham tDCS).

We also estimated maximal training gains during the post-surgery phase by determining the minimum performance during post4-post8 and the maximum performance during post9-post13, as well as % recovery = max (post9-post13) / max (pre1-pre10)*100. Note that % recovery < 100 indicates that the rats could not fully restore pre-surgery performance despite the post-surgery training, whereas % recovery > 100 indicates that rats performed better at the end of the post-surgery training than pre-surgery. Pearson's r correlation was used to analyse whether there was a relationship between the lesion volume and the % recovery.

All results in the text are reported as mean (M) and standard deviation (STD). Error bars in the figure display the standard error of the mean (SEM). The criterion for statistical significance was set to $p < 0.05$ and Tukey's post-hoc test was used to further analyse significant interaction effects. The Shapiro-Wilk test was used to confirm normality of the data and Mauchley's test confirmed that sphericity was not violated. All statistical analyses were performed with Statistica version 11, (StatSoft USA).

RESULTS

Anodal tDCS and sham tDCS groups improved G_s and $G\&T_s$ performance over the course of pre-surgery training (main effect of *Time* G_s: $F_{(9, 135)} = 6.7$, $p < 0.001$ and $G\&T_s$: $F_{(9, 126)} = 4.2$, $p < 0.001$. Learning gains were not significantly different between the anodal tDCS and the sham tDCS groups *Group x Time* interaction G_s: $F_{(9, 135)} = 1.750$, $p = 0.083$ and $G\&T_s$: $F_{(9, 126)} = 1.853$, $p = 0.064$.

Neither maximum pre-surgery performance nor performance on the fourth day post-surgery (post-4) differed significantly between the anodal tDCS and sham tDCS groups ($p = 0.45$; Table 3) and both groups exhibited a highly similar loss of function after surgery as indicated by main effect of *Time* G_s: $F_{(10, 150)} = 10.410$, $p < 0.001$ and $G\&T_s$: $F_{(10, 140)} = 8.366$, $p < 0.001$ (for both G_s and $G\&T_s$ only a non-significant *Group x Time interaction* was found G_s: $F_{(10, 150)} = 1.467$, $p = 0.15$ and $G\&T_s$: $F_{(10, 140)} = 1.51$, $p = 0.13$. These results indicate that both groups performed similarly during the pre-surgery training and that upper limb function was similarly impaired immediately after the experimentally induced stroke (Fig 3A & 3B).
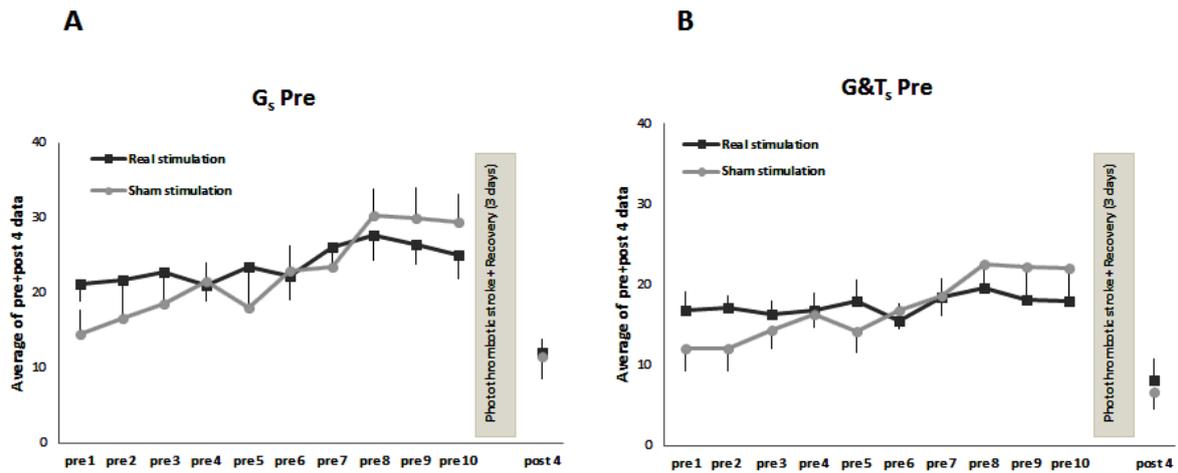
Figure 3: Pre-surgery and post-4 performance for Gs (A) and G&Ts (B), real tDCS group (dark grey squares) and the sham tDCS group (light grey circles). Data are shown as M ± SEM.

Next we analysed upper limb recovery during the post-surgery training. Post-stroke, both groups displayed a reduction in motor performance relative to the pre-stroke baseline. Normalized G_s values (Fig. 4A) showed a performance drop of approximately 60 % after the experimentally induced stroke which was followed by steady recovery of function as indicated by a significant main effect of *Time* $F(9, 135) = 7.18$, $p < 0.001$. Recovery did not differ between the anodal tDCS and sham tDCS groups, main effect of *Group* $F(1, 15) = 0.044$, $p = 0.83$ and the *Group x Time* interaction did not reach significance $F(9, 135) = 1.62$, $p = 0.113$. For $G\&T_s$ there was a main effect of *Time* $F(9, 135) = 5.10$, $p < 0.001$ as well as a significant *Time x Group* interaction $F(9, 135) = 2.02$, $p = 0.040$ (Fig. 4B). However, post-hoc tests revealed no group differences at any of the 10 post-surgery training days. The main effect of *Group* did not reach significance $F(1, 15) = 0.038$, $p = 0.84$.

Next we focussed on G_s performance and inspected recovery gains for the individual rats in form of the minimum G_s observed during the first half of post-surgery training and the maximum G_s observed during the second half, respectively. As can be seen in Figure 4C, there were 3 rats in the anodal tDCS group that hardly performed any grasping movement post-surgery: rat 1 and 2 did not grasp at all and rat 3 had a maximum of 4 grasps. We then evaluated whether this poor post-surgery function might result from variations in lesion size and/or gross lesion location. Figure 4D shows the % recovery of G_s as a function of lesion size in the ipsilesional hemisphere (upper panel) and in the contralesional hemisphere (lower panel). Note that lesion size and location data were only available for 9 rats due to a technical malfunction. It can be seen that the lesion volume of the ipsilesional hemisphere does not predict poor recovery in either group which was further confirmed by a non-significant correlation between lesion size and % recovery (Pearson's $r = -0.55$; $p = 0.124$, calculated across all animals). However, damage in the contralesional hemisphere appears to correlate with

poor grasp performance: all rats that recovered only 12 % or less of their previous function had large and above-average contralesional damage, while all other rats that recovered at least 50 % or more of G_s function had only minor damage in the contralesional hemisphere. Accordingly a correlation revealed a Pearson's r = -0.838; p = 0.005. Equivalent results are obtained when $G\&T_s$ is used as a performance indicator (ipsilesional hemisphere: Pearson's r = -0.426; p = 0.25, and contralesional hemisphere: Pearson's r = -0.78; p = 0.013, calculated across all animals).

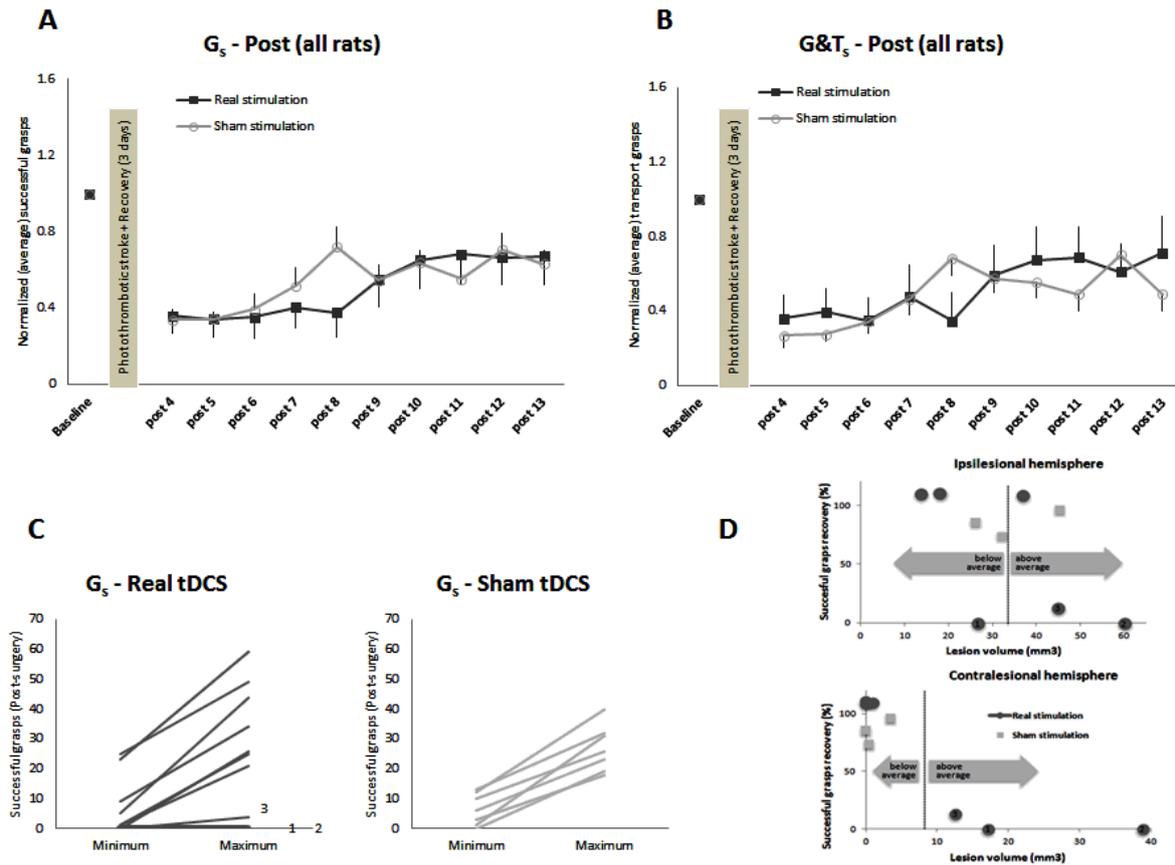**Figure 4:** Performance during post-stroke recovery. Changes in G_s (**A**) and $G\&T_s$ (**B**) relative to pre-stroke baseline. Data are shown over the time course of 10 days, i.e. from day 4 - day 13 post-stroke for the anodal tDCS group (dark grey squares) and the sham tDCS group (light grey circles). **C)** Individual data of post-stroke recovery shown as the minimum and maximum of G_s (see methods for detailed definition) for both groups. The data show an improvement of grasping performance in all rats except for 3 rats in the anodal tDCS group which hardly grasp post-stroke (indicated by numbers 1, 2, 3).

D) Relationship between % recovery and lesion size. Data are shown for 6 rats of the anodal tDCS group (dark grey) and for 3 rats of the sham tDCS group (light grey). The upper panel shows the relationship between % recovery and lesion size in the ipsilesional hemisphere. Note that %recovery larger than 100 indicates that grasping performance at post-10 was better than during the pre-training, i.e. indicating full recovery. The vertical dotted line represents the mean lesion volume across all rats with valid histological data. The lower panel shows the same relationship considering the lesion volume within the contralesional hemisphere. Note that lesion size in the ipsilesional hemisphere does not predict poor recovery in any of the groups whereas large contralesional damage, i.e. rats with lesions that extend across hemispheres do simply not grasp

during the post-lesion period. Three rats (dark grey) have a large bilateral lesion. Only one of them recovers slightly (12%, rat 3) and the other two rats do not recover at all. Data in A and B are shown as M ± SE.

However, due to technical problems we lack the histological examination of 8 rats. Although, we cannot firmly rule out the possibility that also some of these rats had damage in the contralesional hemisphere all of them grasped during the post-recovery phase. With this limitation in mind, we performed a hypothesis-generating analysis after we removed the three non-grasping rats from the data set of the anodal tDCS group. Again, each parameter was analysed separately. Firstly we investigated the post-surgery G_s of the anodal tDCS and sham tDCS groups (Fig 5 and table 4). Post-stroke, both groups displayed a reduction in motor performance relative to the pre-stroke baseline (table 3). Normalized values in figure 5A showed a performance drop of approximately 60 % after the experimentally induced stroke which was followed by a steady recovery in forelimb function as indicated by a significant main effect of *Time* F(9, 108) = 9.69, p < 0.001. Recovery differed between the anodal tDCS and sham tDCS group as indicated by a significant *Group x Time* interaction F(9, 108) = 2.53, p = 0.011. The anodal tDCS group starts to differentiate from the sham tDCS group from the 10th day post-surgery (Post 10) as indicated by post-hoc tests. During the first 5 days of post-stroke training there was no significant difference between anodal and sham tDCS groups F(4, 48) = 1.27, p > 0.05. The same pattern of results was observed for $G\&T_s$, a steady recovery in forelimb function was noticed as shown by a significant *Time* effect F(9, 108) = 6.26, p < 0.001. Recovery differed between anodal tDCS and sham tDCS as indicated by a significant *Group x Time* interaction F(9, 108) = 2.56, p = 0.010 (Fig. 5B).

Even though, a drop in performance of all rats was clearly evident after inducing the experimental stroke, the data show that the animals in the anodal tDCS group recovered quite well reaching almost their pre-surgery level (Fig 5A&B) while the sham tDCS rats remained clearly below. Accordingly, average % recovery values were > 100% in the anodal tDCS group ($G\&T_s$: 121 ± 37 %; G_s: 113 ± 17 %) and < 100 % in the sham tDCS group ($G\&T_s$: 86 ± 22 %; G_s: 79 ± 19 %) and effect size estimations suggest that anodal tDCS had a large treatment effect (Cohen's d $G\&T_s$: 1.14; G_s: 1.89).

The occurrence of $G_{healthy}$ was generally low during the recovery period since the rats did not grasp with the non-affected limb (except for two rats, one grasping once and the other grasping 4 times with the non-affected limb across all post-session).
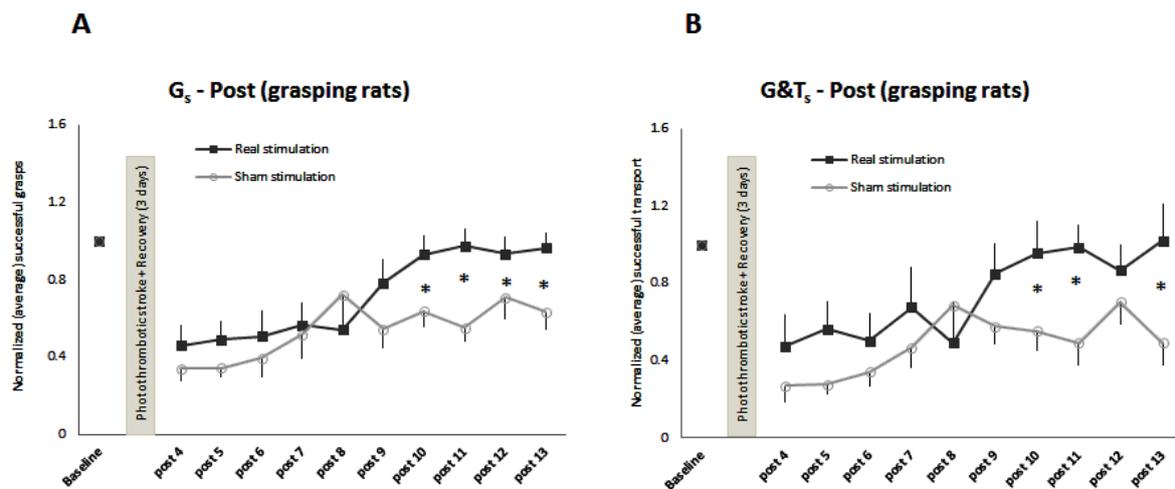
Figure 5: Performance during post-stroke recovery when only grasping rats are considered. Same conventions as in figure 3 A, B., real tDCS group (dark grey squares) and the sham tDCS group (light grey circles). * indicates where *post hoc* tests revealed significant differences of stimulation (p<0.05). Data are shown as M ± SE.

DISCUSSION

The present study tested the feasibility of applying anodal tDCS together with recovery training of reaching and grasping performance in rats following the induction of photothrombotic stroke in primary motor cortex. Our main finding is that the application of 0.05 mA of anodal tDCS to the ipsilesional cortex during 30 min of motor training led to significantly improved recovery when compared to the sham tDCS group. However, it is important to note that the beneficial effect of anodal tDCS only reached significance when we excluded 3 rats from the anodal tDCS group that had large bilateral lesions and performed hardly any grasping movements in the post-period.

To the best of our knowledge, our results are the first combining tDCS with rehabilitation training in a stroke animal model. Our study was designed to mimic human stroke rehabilitation as closely as possible and our data show that it is feasible to apply tDCS while rats are involved in rehabilitation training. Rather than focusing on general aspects of motor coordination that are more typically investigated in rodent studies, we used the pasta matrix reaching task. This task allowed us to determine the effectiveness of anodal tDCS during the training and recovery of reaching and grasping, movements that are often affected by stroke in human and form a crucial part of the rehabilitation process. Given that the sham tDCS group also recovered limb use to some extent, our results highlight the benefits of frequent and targeted rehabilitation training. This type of practice has been shown to trigger use-dependent plasticity resulting from LTP in primary motor cortex when

healthy rats are tested (Rioult-Pedotti et al., 2000; Rioult-Pedotti et al., 1998). Our data suggest that anodal tDCS might be useful as an adjuvant therapy to boost the benefit of post-stroke rehabilitation training. However, it is important to keep in mind that significant differences between the anodal tDCS and sham tDCS group were only observed after 3 non-grasping rats were removed from the anodal tDCS group. This is generally in line with the available data in healthy human volunteers showing that tDCS is only efficient in facilitating behavioural markers of learning when it is combined with executing a motor task (Antal et al., 2004; Reis et al., 2013; Saucedo Marquez et al., 2013; Tanaka et al., 2009). Moreover our results suggests that functional recovery might not be solely achieved within the ispilesional hemisphere but might additionally rely on midline areas (including cingulate cortex) and on contralesional sensorimotor cortex (Lindau et al., 2014). Interhemispheric exchange during recovery from stroke has been reported in human patients (Carter et al., 2010) as well as in animal models (Greifzu et al., 2011; Mohajerani et al., 2011) so that the contralesional hemisphere is strongly activated when the paretic limb is moved. However, this strong contralesional activity is usually strongest early after the insult and is gradually reduced when forelimb function is recovered. Accordingly, one hallmark of successful recovery from human stroke is that the ipsilesional hemisphere regains control over the paretic limb (for a review see Grefkes & Ward, (2013)). This might explain why we found virtually no recovery when the stroke affected a substantial part of sensorimotor areas in both hemispheres. Note however, that bilateral cortical lesions spanning both sensorimotor cortices are rarely seen in human stroke survivors.

In summary, our study shows that applying tDCS in rats during motor training is feasible and when lesions are restricted to one hemisphere such that rats can actually perform the grasping training, anodal tDCS over M1 might facilitate functional recovery. In fact, animals in the anodal tDCS group reached and sometimes even exceeded pre-surgery grasping proficiency. Even though complete recovery is not uncommon in rat models of stroke (particularly when the lesion is relatively small as in our case) anodal tDCS boosted recovery as a function of time and the size of this effect was large in our study. However, future studies using an improved design are necessary to corroborate our results. In addition to task selection, maximising the relevance of work conducted in animal models to human stroke rehabilitation also requires the consideration of other aspects of the experimental design. In particular, anodal tDCS current density is an important parameter that is likely to influence the effectiveness of non-invasive brain stimulation. In human tDCS studies, current densities typically range from $0.28 - 0.80$ A/m^2 (Brunoni et al., 2012). Although we used a significantly higher current density (15.9 A/m^2), it is low compared to stimulation parameters typically used in rodent studies (see also Table 1) with the exception of Kim et al., 2010 who used unusually large electrodes (10 mm diameter) that must have covered most of the rat head. It is important that future studies in

rodents attempt to use current densities that are comparable to those tolerated in human subjects. With regard to the application of tDCS itself, in animals electrodes are placed directly over the skull reducing tissue shunting and current dispersion (Miranda et al., 2009; Sadleir et al., 2010). While tDCS in human can presently not overcome this limitation when applied transcranially and in a minimally invasive way, it is an important consideration when comparing the effectiveness of tDCS between human and animal models.

Another parameter that determines the effectiveness of post stroke training and tDCS application is onset time. While it is more difficult to match rehabilitation onset time in animal and human work due to developmental and lifespan differences, it has clearly been an important factor in determining the effectiveness of post stroke rehabilitation in animal models. Yoon et al., (2012) reported that the application of tDCS in rats was more effective when started either 7 or 11 days post stroke compared to 1 day post stoke, suggesting that anodal tDCS has no benefit when applied very early after the insult. However, it is also critical not to start rehabilitation protocols too late because early interventions in general have a more beneficial effect on long-term behavioural outcomes (Adkins & Jones, 2005; Biernaskie et al., 2004; Farrell et al., 2001). Biernaskie et al., (2004) reported that initiating rehabilitation in animals at 5 days-post stroke induces significantly greater functional recovery and enhanced structural plasticity compared to initiating rehabilitation at 30 days post stroke. Although there is still no consensus on the optimal time window to begin rehabilitation, it is known that an injured brain is susceptible to stimulation in the subacute phase (Kim et al., 2009). Almost all recovery occurs in the first month after stroke in rodents (Biernaskie et al., 2004; Murphy & Corbett, 2009) and in the first three months in humans (Krakauer et al., 2012; Prabhakaran et al., 2015). In the present study we started the concurrent application of tDCS with training 4 days after stroke onset. Although we only observed a significant difference between anodal and sham tDCS groups after 6 training-sessions (i.e. at day 9 post-stroke), it remains possible that anodal tDCS applied during the first 5 sessions of training contributed to this effect. While it is not possible to determine whether or not this was the case with the current design, further work is required to determine the optimal onset for post stroke training and tDCS application. Finally, the present study has some important limitations. First, the sample size was greatly reduced due to the exclusion of animals for various reasons and we were not able to perform histology on all rats reported here. Although animal models offer the potential for a more homogeneous population than can usually be tested in humans, there was some degree of inter-individual variation in the location and size of lesion resulting from the photothrombotic stroke. In this regard, we note that this is the first experiment of its kind conducted in our lab. Future studies would certainly benefit from MRI examinations performed after the experimental stroke has been applied but before animals are assigned to different training groups in order to better match lesion characteristics across groups.

Unfortunately, practical constraints prevented to replace rats that had to be excluded from analysis. However, a post-hoc power calculation based on the % recovery of the successful grasping actions revealed a power = 0.92, indicating that the final sample size of our study was appropriate to detect statistically significant effects.

Our work departed from the premise that tDCS is most efficient when combined with rehabilitation training. In order to support this view experimentally, future studies should include control groups that do not engage in specific rehabilitation training but receive either anodal tDCS or sham tDCS during rest to test whether the simultaneous application of tDCS and training produces effects over and above those found for each intervention alone. Another important limitation is the age of the rats used in the study. Animals arrived in the lab at approximately 6 − 7 weeks of age, which is relatively young. It is likely that tDCS effects are not the same in young and old subjects (Yoon et al., 2012), and also that recovery from injury might differ, for example, due to reduced neuroplasticity in the elderly (Datta et al., 2012; Song et al, 2011). Furthermore, given that the majority of stroke patients are elderly, older animals should be used in future studies to improve the relevance of the results. Although animal studies testing rehabilitation interventions should try to optimise their design for maximum transfer to the human clinical setting, it is important to keep in mind that animal models do not completely duplicate the complexity of stroke in human (Kleim et al, 2007).

In conclusion, we showed that the application of anodal tDCS during post stroke training on a reaching and grasping task in rats is feasible and that it was beneficial to upper limb recovery when compared to a sham tDCS group. We propose that it is essential to apply anodal tDCS over primary motor cortex together with recovery training, however, in order to substantiate this argument one needs a control group where the effect of anodal tDCS versus sham tDCS is tested without concurrent motor practice. The daily application of anodal tDCS to the ipsilesional cortex from day 4-day 13 post-stroke resulted in a significant improvement in the number of successful reaches and grasps when compared to sham stimulation. Our results add to the growing body of evidence suggesting that tDCS is a promising adjuvant therapy to facilitate motor recovery following stroke. More importantly, the availability of an animal model that can be used to closely mimic recovery training in healthy humans opens new avenues for gaining more mechanistic understanding of the underlying principles. This will be important to improve tDCS stimulation protocols in order to maximize motor recovery after stroke.

	G_s Anodal	G_s Sham	G_s p-value	$G\&T_s$ Anodal	$G\&T_s$ Sham	$G\&T_s$ p-value
maxPre-stroke	32 ± 9.27	35 ± 8.74	0.45	24 ± 9.20	25 ± 5.75	0.75
Post 4-stroke	12 ± 10.95	11 ± 6.18	0.90	8 ± 8.09	6 ± 5.55	0.68

Table 3: Performance pre-surgery (maxPRE) and performance at post-4 (i.e. the first training after the experimental lesion) when all rats (n=17) are included in the analysis. P-values were derived from independent t-tests comparing the anodal tDCS and the sham tDCS group. Data are shown as M ± STD.

	G_s Anodal	G_s Sham	G_s p-value	$G\&T_s$ Anodal	$G\&T_s$ Sham	$G\&T_s$ p-value
Pre-stroke	32 ± 11.20	35 ± 8.74	0.64	25 ± 11.12	25 ± 5.75	0.88
Post 4-stroke	15 ± 10.87	11 ± 6.18	0.41	10 ± 8.17	6 ± 5.55	0.28

Table 4: Performance pre-surgery (maxPRE) and performance at post-4 (i.e. the first training after the experimental lesion) when only rats (n=14) with mainly unilateral lesions were included. P-values were derived from independent t-tests comparing the anodal tDCS and the sham tDCS group. Data are shown as M ± STD.

Acknowledgment

Reference

Adkins, D. L., & Jones, T. a. (2005). D-Amphetamine enhances skilled reaching after ischemic cortical lesions in rats. *Neuroscience Letters*, *380*, 214–218. http://doi.org/10.1016/j.neulet.2005.01.036

Antal, A., Nitsche, M. a., Kincses, T. Z., Kruse, W., Hoffmann, K. P., & Paulus, W. (2004). Facilitation of visuo-motor learning by transcranial direct current stimulation of the motor and extrastriate visual areas in humans. *European Journal of Neuroscience*, *19*, 2888–2892. http://doi.org/10.1111/j.1460-9568.2004.03367.x

Ballermann, M., Metz, G. a S., McKenna, J. E., Klassen, F., & Whishaw, I. Q. (2001). The pasta matrix reaching task: A simple test for measuring skilled reaching distance, direction, and dexterity in rats. *Journal of Neuroscience Methods*, *106*, 39–45. http://doi.org/10.1016/S0165-0270(01)00326-0

Ballermann, M., Tompkins, G., & Whishaw, I. Q. (2000). Skilled forelimb reaching for pasta guided by tactile input in the rat as measured by accuracy, spatial adjustments, and force. *Behavioural Brain Research*, *109*(1), 49–57. http://doi.org/10.1016/S0166-4328(99)00164-3

Benowitz, L. I., & Routtenberg, A. (1997). GAP-43: An intrinsic determinant of neuronal development and plasticity. *Trends in Neurosciences*, *20*(2), 84–91. http://doi.org/10.1016/S0166-2236(96)10072-2

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(5), 1245–1254. http://doi.org/10.1523/JNEUROSCI.3834-03.2004

Brunoni, A. R., Nitsche, M. a., Bolognini, N., Bikson, M., Wagner, T., Merabet, L., … Fregni, F. (2012). Clinical research with transcranial direct current stimulation (tDCS): Challenges and future directions. *Brain Stimulation*, *5*(3), 175–195. http://doi.org/10.1016/j.brs.2011.03.002

Carter, A. R., Astafiev, S. V., Lang, C. E., Connor, L. T., Rengachary, J., Strube, M. J., … Corbetta, M. (2010). Resting interhemispheric functional magnetic resonance imaging connectivity predicts performance after stroke. *Annals of Neurology*, *67*(3), 365–375. http://doi.org/10.1002/ana.21905

Datta, A., Truong, D., Minhas, P., Parra, L. C., & Bikson, M. (2012). Inter-individual variation during transcranial direct current stimulation and normalization of dose using MRI-derived computational models. *Frontiers in Psychiatry*, *3*(October), 1–8.

http://doi.org/10.3389/fpsyt.2012.00091

Dietrich, W. D., Ginsberg, M. D., Busto, R., & Watson, B. D. (1986). Photochemically induced cortical infarction in the rat. 2. Acute and subacute alterations in local glucose utilization. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *6*, 195–202. http://doi.org/10.1038/jcbfm.1986.32

Farrell, R., Evans, S., & Corbett, D. (2001). Environmental enrichment enhances recovery of function but exacerbates ischemic cell death. *Neuroscience*, *107*(4), 585–592. http://doi.org/10.1016/S0306-4522(01)00386-4

Fregni, F., Boggio, P. S., Mansur, C. G., Wagner, T., Ferreira, M. J. L., Lima, M. C., … Pascual-Leone, A. (2005). Transcranial direct current stimulation of the unaffected hemisphere in stroke patients. *Neuroreport*, *16*(14), 1551–1555. http://doi.org/10.1097/01.wnr.0000177010.44602.5e

Fritsch, B., Reis, J., Martinowich, K., Schambra, H. M., Ji, Y., Cohen, L. G., & Lu, B. (2011). Direct current stimulation promotes BDNF-dependent synaptic plasticity: Potential implications for motor learning. *NIH Public Access*, *66*(2), 198–204. http://doi.org/10.1016/j.neuron.2010.03.035.Direct

Grefkes, C., & Ward, N. S. (2013). Cortical Reorganization After Stroke: How Much and How Functional? *The Neuroscientist*, *20*(1), 56–70. http://doi.org/10.1177/1073858413491147

Greifzu, F., Schmidt, S., Schmidt, K.-F., Kreikemeier, K., Witte, O. W., & Löwel, S. (2011). Global impairment and therapeutic restoration of visual plasticity mechanisms after a localized cortical stroke. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(37), 15450–15455. http://doi.org/10.1073/pnas.1016458108

Hesse, S., Werner, C., Schonhardt, E. M., Bardeleben, A., Jenrich, W., & Kirker, S. G. B. (2007). Combined transcranial direct current stimulation and robot-assisted arm training in subacute stroke patients: a pilot study. *Restorative Neurology and Neuroscience*, *25*, 9–15. http://doi.org/10.1177/1545968311413906

Hummel, F. C., & Cohen, L. G. (2006). Non-invasive brain stimulation: a new strategy to improve neurorehabilitation after stroke? *The Lancet Neurology*, *5*(August), 708–712. http://doi.org/10.1016/S1474-4422(06)70525-7

Hummel, F., Celnik, P., Giraux, P., Floel, A., Wu, W. H., Gerloff, C., & Cohen, L. G. (2005). Effects of non-invasive cortical stimulation on skilled motor function in chronic stroke. *Brain*, *128*, 490–499. http://doi.org/10.1093/brain/awh369

Jiang, T., Xu, R. X., Zhang, a. W., Di, W., Xiao, Z. J., Miao, J. Y., … Fang, Y. N. (2012). Effects of transcranial direct current stimulation on hemichannel pannexin-1 and neural plasticity in rat model of cerebral infarction. *Neuroscience*, *226*, 421–426. http://doi.org/10.1016/j.neuroscience.2012.09.035

Kerr, A. L., & Tennant, K. a. (2014). Compensatory Limb Use and Behavioral Assessment of Motor Skill Learning Following Sensorimotor Cortex Injury in a Mouse Model of Ischemic Stroke. *Journal of Visualized Experiments*, (July), 1–7. http://doi.org/10.3791/51602

Kim, D. Y., Ohn, S. H., Yang, E. J., Park, C.-I., & Jung, K. J. (2009). Enhancing motor performance by anodal transcranial direct current stimulation in subacute stroke patients. *American Journal of Physical Medicine & Rehabilitation / Association of Academic Physiatrists*, *88*, 829–836. http://doi.org/10.1097/PHM.0b013e3181b811e3

Kim, S. J., Kim, B. K., Ko, Y. J., Bang, M. S., Kim, M. H., & Han, T. R. (2010). Functional and histologic changes after repeated transcranial direct current stimulation in rat stroke model. *Journal of Korean Medical Science*, *25*, 1499–1505. http://doi.org/10.3346/jkms.2010.25.10.1499

Kleim, J. a, Boychuk, J. a, & Adkins, D. L. (2007). Rat models of upper extremity impairment in stroke. *ILAR Journal / National Research Council, Institute of Laboratory Animal Resources*, *48*, 374–384.

Kolominsky-Rabas, P. L., Weber, M., Gefeller, O., Neundoerfer, B., & Heuschmann, P. U. (2001). Epidemiology of Ischemic Stroke Subtypes, 2735–2740.

Krakauer, J. W., Carmichael, S. T., Corbett, D., & Wittenberg, G. F. (2012). Getting Neurorehabilitation Right: What Can Be Learned From Animal Models? *Neurorehabilitation and Neural Repair*, *26*, 923–931. http://doi.org/10.1177/1545968312440745

Labat-gest, V., & Tomasi, S. (2013). Photothrombotic ischemia: a minimally invasive and reproducible photochemical cortical lesion model for mouse stroke studies. *Journal of Visualized Experiments : JoVE*, (76), 1–6. http://doi.org/10.3791/50370

Lefebvre, S., Laloux, P., Peeters, a, Desfontaines, P., Jamart, J., & Vandermeeren, Y. (2012). Dual-tDCS Enhances Online Motor Skill Learning and Long-Term Retention in Chronic Stroke Patients. *Frontiers in Human Neuroscience*, *6*(January), 343. http://doi.org/10.3389/fnhum.2012.00343

Liebetanz, D., Fregni, F., Monte-Silva, K. K., Oliveira, M. B., Amâncio-dos-Santos, Â., Nitsche, M. a., & Guedes, R. C. a. (2006). After-effects of transcranial direct current stimulation (tDCS) on cortical spreading depression. *Neuroscience Letters*, *398*, 85–90.

http://doi.org/10.1016/j.neulet.2005.12.058

Liebetanz, D., Koch, R., Mayenfels, S., König, F., Paulus, W., & Nitsche, M. a. (2009). Safety limits of cathodal transcranial direct current stimulation in rats. *Clinical Neurophysiology*, *120*(6), 1161–1167. http://doi.org/10.1016/j.clinph.2009.01.022

Lindau, N. T., Bänninger, B. J., Gullo, M., Good, N. a., Bachmann, L. C., Starkey, M. L., & Schwab, M. E. (2014). Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. *Brain*, *137*(3), 739–756. http://doi.org/10.1093/brain/awt336

Miranda, P. C., Faria, P., & Hallett, M. (2009). What does the ratio of injected current to electrode area tell us about current density in the brain during tDCS? *Clinical Neurophysiology*, *120*(6), 1183–1187. http://doi.org/10.1016/j.clinph.2009.03.023

Mohajerani, M. H., Aminoltejari, K., & Murphy, T. H. (2011). Targeted mini-strokes produce changes in interhemispheric sensory signal processing that are indicative of disinhibition within minutes. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(22), E183–E191. http://doi.org/10.1073/pnas.1101914108

Moon, S. K., Alaverdashvili, M., Cross, A. R., & Whishaw, I. Q. (2009). Both compensation and recovery of skilled reaching following small photothrombotic stroke to motor cortex in the rat. *Experimental Neurology*, *218*(1), 145–153. http://doi.org/10.1016/j.expneurol.2009.04.021

Murphy, T. H., & Corbett, D. (2009). Plasticity during stroke recovery: from synapse to behaviour. *Nature Reviews. Neuroscience*, *10*(DECEmbEr), 861–872. http://doi.org/10.1038/nrn2735

Nitsche, M. a, & Paulus, W. (2000). Excitability changes induced in the human motor cortex by weak transcranial direct current stimulation. *The Journal of Physiology*, *527 Pt 3*, 633–639. http://doi.org/10.1111/j.1469-7793.2000.t01-1-00633.x

Nitsche, M. a, & Paulus, W. (2001). Sustained excitability elevations induced by transcranial DC motor cortex stimulation in humans. *Neurology*, *57*(10), 1899–1901. http://doi.org/10.1212/WNL.57.10.1899

Notturno, F., Pace, M., Zappasodi, F., Cam, E., Bassetti, C. L., & Uncini, A. (2014). Neuroprotective effect of cathodal transcranial direct current stimulation in a rat stroke model. *Journal of the Neurological Sciences*, *342*(1-2), 146–151. http://doi.org/10.1016/j.jns.2014.05.017

Paxinos G., W. C. (2007). The rat brain in stereotaxix coordinates, 6th edn. Elsevier Science, San Diego, USA.

Peruzzotti-Jametti, L., Cambiaghi, M., Bacigaluppi, M., Gallizioli, M., Gaude, E., Mari, S., … Leocani, L.

(2013). Safety and efficacy of transcranial direct current stimulation in acute experimental ischemic stroke. *Stroke*, *44*, 3166–3174. http://doi.org/10.1161/STROKEAHA.113.001687

Pevsner, P. H., Eichenbaum, J. W., Miller, D. C., Pivawer, G., Eichenbaum, K. D., Stern, A., … Koutcher, J. a. (2001). A photothrombotic model of small early ischemic infarcts in the rat brain with histologic and MRI correlation. *Journal of Pharmacological and Toxicological Methods*, *45*(2001), 227–233. http://doi.org/10.1016/S1056-8719(01)00153-8

Prabhakaran, S., Zarahn, E., Riley, C., Speizer, A., Chong, J. Y., Lazar, R. M., … Krakauer, J. W. (2015). Inter-individual variability in the capacity for motor recovery after ischemic stroke. *Neurorehabilitation and Neural Repair*, *22*, 64–71. http://doi.org/10.1177/1545968307305302

Reis, J., Fischer, J. T., Prichard, G., Weiller, C., Cohen, L. G., & Fritsch, B. (2013). Time- but Not Sleep- Dependent Consolidation of tDCS-Enhanced Visuomotor Skills. *Cerebral Cortex (New York, N.Y. : 1991)*, 1–9. http://doi.org/10.1093/cercor/bht208

Rioult-Pedotti, M. S., Friedman, D., & Donoghue, J. P. (2000). Learning-induced LTP in neocortex. *Science (New York, N.Y.)*, *290*(2000), 533–536. http://doi.org/10.1126/science.290.5491.533

Rioult-Pedotti, M. S., Friedman, D., Hess, G., & Donoghue, J. P. (1998). Strengthening of horizontal cortical connections following skill learning. *Nature Neuroscience*, *1*(3), 230–234. http://doi.org/10.1038/678

Rossi, C., Sallustio, F., Di Legge, S., Stanzione, P., & Koch, G. (2013). Transcranial direct current stimulation of the affected hemisphere does not accelerate recovery of acute stroke patients. *European Journal of Neurology : The Official Journal of the European Federation of Neurological Societies*, *20*, 202–4. http://doi.org/10.1111/j.1468-1331.2012.03703.x

Rueger, M. A., Keuters, M. H., Walberer, M., Braun, R., Klein, R., Sparing, R., … Schroeter, M. (2012). Multi-session transcranial direct current stimulation (tDCS) Elicits inflammatory and regenerative processes in the rat brain. *PLoS ONE*, *7*(8). http://doi.org/10.1371/journal.pone.0043776

Sadleir, R. J., Vannorsdall, T. D., Schretlen, D. J., & Gordon, B. (2010). Transcranial direct current stimulation (tDCS) in a realistic head model. *NeuroImage*, *51*(4), 1310–1318. http://doi.org/10.1016/j.neuroimage.2010.03.052

Sattler, V., Acket, B., Raposo, N., Albucher, J.-F., Thalamas, C., Loubinoux, I., … Simonetta-Moreau, M. (2015). Anodal tDCS Combined With Radial Nerve Stimulation Promotes Hand Motor Recovery in the Acute Phase After Ischemic Stroke. *Neurorehabilitation and Neural Repair*.

http://doi.org/10.1177/1545968314565465

Saucedo Marquez, C. M., Zhang, X., Swinnen, S. P., Meesen, R., & Wenderoth, N. (2013). Task-specific effect of transcranial direct current stimulation on motor learning. *Frontiers in Human Neuroscience*, *7*(July), 333. http://doi.org/10.3389/fnhum.2013.00333

Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L., & Bland, S. T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology*, *39*, 777–787. http://doi.org/10.1016/S0028-3908(00)00005-8

Schmidt, A., Hoppen, M., Strecker, J.-K., Diederich, K., Schäbitz, W.-R., Schilling, M., & Minnerup, J. (2012). Photochemically induced ischemic stroke in rats. *Experimental & Translational Stroke Medicine*, *4*, 13. http://doi.org/10.1186/2040-7378-4-13

Song, C., Schwarzkopf, D. S., Kanai, R., & Rees, G. (2011). Reciprocal anatomical relationship between primary sensory and prefrontal cortices in the human brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *31*(26), 9472–9480. http://doi.org/10.1523/JNEUROSCI.0308-11.2011

Stagg, C. J., Jayaram, G., Pastor, D., Kincses, Z. T., Matthews, P. M., & Johansen-Berg, H. (2011). Polarity and timing-dependent effects of transcranial direct current stimulation in explicit motor learning. *Neuropsychologia*, *49*(5), 800–804. http://doi.org/10.1016/j.neuropsychologia.2011.02.009

Tanaka, S., Hanakawa, T., Honda, M., & Watanabe, K. (2009). Enhancement of pinch force in the lower leg by anodal transcranial direct current stimulation. *Experimental Brain Research*, *196*, 459–465. http://doi.org/10.1007/s00221-009-1863-9

Wachter, D., Wrede, A., Schulz-Schaeffer, W., Taghizadeh-Waghefi, A., Nitsche, M. a., Kutschenko, A., … Liebetanz, D. (2011). Transcranial direct current stimulation induces polarity-specific changes of cortical blood perfusion in the rat. *Experimental Neurology*, *227*, 322–327. http://doi.org/10.1016/j.expneurol.2010.12.005

Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., & Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Annals of Neurology*. http://doi.org/10.1002/ana.410170513

Yoon, K. J., Oh, B. M., & Kim, D. Y. (2012). Functional improvement and neuroplastic effects of anodal transcranial direct current stimulation (tDCS) delivered 1 day vs. 1 week after cerebral ischemia

in rats. *Brain Research*, *1452*, 61–72. http://doi.org/10.1016/j.brainres.2012.02.062

Zeiler SR and Krakauer JW. (2012). The interaction between training and plasticity in the post-stroke brain. *Changes*, *29*(6), 997–1003. http://doi.org/10.1016/j.biotechadv.2011.08.021.Secreted

Chapter five

General discussion

The aim of this chapter is to provide a general discussion of all studies included in this project. First, we will briefly summarize the main findings of the different experiments. We will then discuss which of the methods used in this specific PhD project are promising tools for facilitating neuroplasticity. Finally, we will provide suggestions for future research directions.

Overview of results

Chapter 2: **The influence of reward and punishment on use-dependent plasticity**

Research question: Does training under rewarded versus punished conditions influence use-dependent plasticity?

Methods: Ballistic thumb movements (thumb flexion) were performed as fast as possible. Peak movement velocity was estimated to quantify motor learning at the behavioural level. Transcranial Magnetic Stimulation was used to determine changes in corticomotor excitability of the thumb flexor in response to practice.

Results: Behavioural data indicate that the reward and punishment groups learned and retained the motor task equally well. Only the punishment group showed an increase in corticomotor excitability from baseline to post training while the reward group exhibited even a slight, initial decrease.

Conclusion: Behavioural markers of use-dependent plasticity were not differentially modulated by monetary reward or punishment which might result from the nature of the task. Surprisingly, training under rewarded but not under punished conditions prevented the increase of corticomotor excitability which is typically observed in this task. This finding was highly unexpected and requires further evaluation to be fully understood.

Chapter 3: **Anodal tDCS over the primary motor cortex facilitates long-term memory formation reflecting use-dependent plasticity**

Research question: Does anodal tDCS over the primary motor cortex improve consolidation of motor memories which are formed as the result of repetitive practice.

Methods: Using a double-blind cross-over design, anodal tDCS (1mA) or sham tDCS was applied for 20 min over primary motor cortex (M1) while subjects practiced to flex their thumb as fast as possible.

Results: Retention performance was significantly better when anodal tDCS was applied during training than when sham tDCS was applied during training. However, significance was only reached when retention was tested one week later.

Conclusion: Anodal tDCS facilitates long-term memory formation in motor cortex reflecting use-dependent plasticity but only when tested during retention 1 day and 1 week later. Our data suggests that a single training session with tDCS makes M1 more sensitive to undergo neuroplastic changes during subsequent training.

Chapter 4: **The effect of tDCS on motor recovery in an animal model of stroke**

Research question: Does anodal tDCS over ipsilesional M1 improve the recovery of reaching and grasping performance after a photothrombotic stroke in rats?

Methods: Anodal tDCS of 0.05 mA was applied for 30 min to the ipsilesional M1 while animals were trained on a pasta matrix reaching task for 10 days after the surgery, inducing photothrombotic stroke. A control group underwent the identical protocol but received sham tDCS.

Results: The application of anodal tDCS led to significantly improved recovery when compared to the sham tDCS group. However, the beneficial effects of anodal tDCS only reached statistical significance when we excluded rats that practiced the grasping task only occasionally because of large bilateral lesions.

Conclusion: We showed that the application of anodal tDCS during post-stroke training on a reaching and grasping task in rats is feasible and that it was beneficial to upper limb recovery.

In the first chapters of this thesis we tested the effect of two potential neuromodulators, i.e. reward/punishment and anodal tDCS over M1, while healthy volunteers repetitively practiced a simple movement. This paradigm serves as a model of use-dependent plasticity (Classen et al., 1998; Rosenkranz et al., 2007) a phenomenon that drives neuroplastic changes for every form of motor learning requiring repetitive practice (Krakauer & Mazzoni, 2011). In particular, use-dependent plasticity might be an essential element of rehabilitation training, which is based on a high number of repetitions (Buma et al., 2013). Using this model we demonstrated in chapter 2 that use-dependent plasticity in response to motor training is only minimally modulated by practice under rewarded versus punished conditions while we found a clear effect of anodal tDCS applied over M1 on long-term memory formation (chapter 3). Based on these results, we opted to investigate the effects of anodal tDCS in a rat model (chapter 4) and tested its efficiency during upper limb recovery early after a photothrombotic stroke. Since previous studies tested the effect of anodal tDCS on recovery after stroke only while rats were anesthetized we aimed to develop an animal model for applying anodal tDCS during motor training, i.e. similar to its application in human stroke survivors. The rehabilitation training consisted of a repetitive reaching and grasping task, a paradigm that has been used previously to demonstrate use-dependent plasticity in rodents (Rioult-Pedotti et al., 2000; Rioult-Pedotti et al., 1998). Our main result was that rats receiving anodal tDCS performed better during training compared to rats receiving sham tDCS. However, significance was only reached when the analysis was restricted to rats that actively performed the training.

Modulation of neuroplasticity: Reward and punishment

Previous work has shown that reward and punishment are important motivators for human and animal behaviour (Daw et al., 2002). More recent studies have further demonstrated that positive and negative feedback have dissociable effects on motor adaptation (Galea et al., 2015), procedural motor learning (Wächter et al., 2009) and motor skill learning (Abe et al., 2011). This work has shown that rewarding good performance is beneficial for the retention of different motor tasks when compared to a punishment or a control group. Here we tested very similar reward/punishment schemes while we induced use-dependent plasticity through repetitive motor practice. Even though our study design was similar to the previous work we could not reproduce the results, as can be seen from highly similar PeakV for the retention tests. Our data revealed no evidence suggesting that the behavioural practice in our task was differentially influenced by rewarding or punishing subjects during training. We hypothesized that these different results are caused by the specific motor task used for our experiment. Note, however, that we only performed a single day training session in

healthy subjects thus we cannot exclude that training under rewarded and/or punished conditions might induce a measurable effect when performed over multiple sessions. It is also possible that the induced effects might be larger in a patient population. Finally, we cannot rule out that both conditions, i.e. monetary reward and punishment facilitated learning relative to a control condition.

Nevertheless, our first experimental study revealed no evidence that monetary reward facilitates motor learning. Thus even though the retention of motor memory depends on M1 (Hadipour-Niktarash et al., 2007), and dopaminergic inputs to M1 have been shown to be crucial for use-dependent plasticity in motor cortex (Hosp & Luft, 2013) the attempt to activate this pathway via our behavioural manipulation was unsuccessful. It is possible, however, that direct pharmacological interventions are better suited to facilitate motor learning and recovery by activating the dopaminergic system and respective clinical trials are currently underway (Bhakta et al., 2014).

Transcranial Direct Current Stimulation in healthy volunteers

Neuroplasticity can be modulated by non-invasive brain stimulation techniques such as transcranial Direct Current Stimulation (tDCS). Numerous studies have tested the effects of tDCS on healthy young volunteers, demonstrating that anodal tDCS over primary motor cortex (M1) combined with motor practice facilitates motor learning (Boggio et al., 2006; Orban de Xivry & Shadmehr, 2014). Its beneficial effects have also been demonstrated when applied during a single training session (Kantak et al., 2012; Tecchio et al., 2010). The results of our study (chapter 3) are in line with the findings in the literature.

Previous research strongly suggests that anodal tDCS over M1 acts on cellular pathways that mediate use-dependent plasticity and should therefore facilitate learning. In our study we tested this hypothesis by applying anodal tDCS during a single training session of ballistic thumb movement task which was followed by several retention tests that were executed 30 min, 24 hours and one week after practice had finished. We quantified use-dependent plasticity by changes of movement kinematics (thumb velocity).

Training resulted in a reliable increase in thumb flexion peak velocity and next we investigated whether training with anodal tDCS influenced performance gains or retention differently than training with sham tDCS. Our main result is that retention performance was significantly better when anodal tDCS was applied during training when compared to training with sham tDCS. Here we could speculate that subjects who received anodal tDCS underwent an accelerated learning process. Since learning curves are generally exponential, accelerated learning is indicated by larger gains in the first session than in the second. This was not the case for the subjects who first received sham tDCS and

exhibited moderate gains during the first training. However, when they returned for the second session a few months later there was still enough room for improvement when training was executed with anodal tDCS. However, it is important to note that the differential effect of anodal versus sham tDCS was not observable during training but only at the retention tests executed at the next day or one week after training. Statistical significance was only reached when retention was tested one week after training, however, the size of this effect was large (Cohen's d = 1.01) and consistent across subjects.

Our finding that anodal tDCS facilitated motor memory formation, but that its beneficial effect was mainly expressed in a retention test, is in line with previous work. In particular Reis et al., (2013) observed that when tDCS was applied to M1 during visuo-motor adaptation, performance benefits were only found 3 h after the end of training. Similar to the present study they also found that no additional performance gains were observed when retention was tested after a single night of sleep, suggesting that the beneficial effect of anodal tDCS on memory consolidation is not sleep dependent. Moreover, our results are in line with previous work (Reis et al., 2013; Saucedo Marquez et al., 2013), in particular Reis et al., (2013) reported that beneficial effects were not found when stimulation was applied after practice, suggesting that the simultaneous application of anodal tDCS and practice triggers subsequent processes important for motor memory formation. Our study confirms and extends this research by demonstrating that anodal tDCS modulates the long-term effects of use-dependent plasticity, a phenomenon that is believed to be mediated by strengthening synapses via LTP-like process (Bütefisch et al., 2000; Classen et al., 1998; Rosenkranz et al., 2007).

In line with these findings is also the study of Stagg et al., (2011) showing that the application of anodal tDCS during an explicit sequence-learning task was associated with faster learning. By contrast, the application of tDCS prior to task performance led to slower learning. These results confirm the idea that tDCS applied concurrently with motor learning is more efficient than applying it prior to the behavioural task. We used a double-blind within-subject cross-over design to reduce the influence of inter-individual differences, which can vary substantially and might result from the genetic background of the individual (Missitzi et al., 2013), since individual genotype might influence the effect of tDCS. For example, brain-derived neurotrophic factor (BDNF) appears to influence the response to tDCS in healthy participants (Teo et al., 2014) and animals (Fritsch et al., 2011).

Also, inter-individual differences in cranial and brain anatomy can influence the impact of tDCS differing substantially across subjects (Datta et al., 2012; Li et al., 2015). While topographical organization of primary motor cortex is relatively consistent across humans (Nudo, 2013), variability exists in the flow of current through the cortex. This variability exists even when the same stimulation intensity is administered across subjects. Anatomical factors do not always have the expected influence, as the current density in the target region might inversely be related to skull

thickness. A recent study has demonstrated that a higher proportion of highly conducting spongy bone in thicker skull areas results in a more complex relationship between skull thickness and current density than previously thought (Opitz et al., 2015). Another important factor to be taken into consideration is electrode montage. The most common electrode montage is placing the anodal electrode with its centre radially over M1 (or the area of interest) and the cathode electrode above eyebrow. However, this montage is not individualized and not very specific (Miranda et al., 2006). In our study we placed the anode over the M1 and the cathode was located over on the ipsilateral shoulder (extracephalic placement).

Nevertheless with some exceptions, tDCS has been shown to be a valuable technique for improving motor learning in healthy participants and even more so in patients with brain injuries such as stroke. One also has to note that we used a very simple model of motor learning (ballistic thumb movements). However, positive effects of anodal tDCS have also been demonstrated when gross motor skills including leg movements were trained (see for example Picelli et al., (2015); Tanaka et al., (2009)).

This makes anodal tDCS a promising, potential tool for modulating neural recovery after stroke.

Until now, however, only few studies have applied tDCS in subacute stroke patients and revealed mixed results (Hesse et al., 2007; Kim et al, 2009; Rossi et al., 2013; Sattler et al., 2015). One reason for being reluctant with applying tDCS early after the insult is that the underlying mechanisms are not yet clear, so that it cannot be excluded that anodal tDCS might even be harmful for the damaged tissue.

In vitro and in vivo animal models have been proven useful for exploring the electrophysiological properties of tDCS (Fritsch et al., 2011) because they allow more in-depth investigation of the cellular and histological modifications induced by tDCS. However, to be truly informative it is important that the animal model mimics current applications in humans as closely as possible.

Transcranial Direct Current Stimulation in a photothrombotic stroke animal model

The most important goal of stroke treatment is the restoration of function in stroke patients. Rodents serve as a model of stroke to identify behavioural deficits and therapeutic treatments which are essential for potential translational applications. Rodent models have provided valuable insight and understanding of the biological basis and functional outcome of stroke. Animal models have shown that functional recovery results either from true recovery/repair of neurons located in the

peri-infarct zone or from reorganization that often happens at the systems level to compensate for lost function (Murphy & Corbett, 2009).

The purpose of our study, in chapter 4, was to develop an animal model, evaluating the treatment effects, of stroke rehabilitation that better mimics tDCS applications in humans using a bedside-to-bench approach. Most importantly, we aimed to develop an animal model where the effect of anodal tDCS over ipsilesional M1 is tested while animals perform goal-directed upper limb training. Therefore, rats were trained on the pasta matrix reaching task, a sensitive behavioural assay that allows the manipulation of limb use in order to mimic human clinical phenomena (Kerr & Tennant, 2014). Reaching and grasping movements are often affected by stroke in human and form a crucial part of the rehabilitation process.

The pasta matrix reaching task assesses skilled forelimb function which is not part of the natural rat behaviour (at least not with the level of dexterity required by the task) but was intensively trained prior to inducing the experimental stroke. Comparing pre-stroke performance to the behaviour post stroke, it provides a sensitive measure of both motor impairment and functional improvement after stroke (Ballermann et al., 2001). However, our analysis does not allow us to distinguish whether rats adopted a compensatory strategy or truly recovered forelimb function (i.e. performed the movement in the same way as prior to stroke). To answer this question one would require a detailed analysis of the grasping kinematics (Whishaw, 2000). Nevertheless this task allowed us to determine the effectiveness of anodal tDCS during the training on regaining function of the pre-trained reaching and grasping movements.

We induced photothrombotic stroke in the primary motor cortex contralateral to the preferred limb. The photothrombotic stroke model induces ischemic damage within a cortical area, through the photo-activation of a light-sensitive dye previously delivered into the blood stream (Dietrich et al., 1986; Labat-gest & Tomasi, 2013; Schmidt et al., 2012; Watson et al., 1985). Location and lesion size can be modulated by altering the duration of light exposure, dye concentration, irradiating intensity and beam position (Schmidt et al., 2012). This approach induces a cortical stroke but has the advantage that the lesion size can be better controlled than in rat models inducing subcortical stroke (for example using the middle cerebral artery occlusion method, (Tamura et al., 1981)). Even though we used a highly controlled protocol to induce the stroke some rats had bilateral lesions and hardly grasped during the post-stroke phase. This indicates that midline areas (including cingulate cortex) and even the contralesional sensorimotor cortex might at least partly contribute to functional recovery of forelimb function in rats (Lindau et al., 2014).

It is likely that the bilateral lesions occurred due to a minimal shift of the cold light source which was not detectable by eye. After injecting Rose Bengal via the femoral vein the animal has to be replaced back on the stereotactic frame to place the cold light source with an aperture of 8 mm diameter upon the skull. Everything has to be done within a 2 min time period and in some animals the cold light source might have moved by a few degrees, thus affecting also the non-targeted hemisphere causing a bi-lateral lesion. For the future experiments this part need to be improved by optimizing the cold light source aperture and/or fixation. Moreover it might be useful to acquire an anatomical magnetic resonance image prior to training so that lesion size and location can be properly identified. This, however, was not possible in the present study. Overall, the occurrence of bilateral damage shows how difficult it is to induce a controlled lesion in rodents, particularly, since this PhD project was the first to implement the photothrombotic stroke model in the laboratory.

Our main finding was that application of anodal tDCS to the ipsilesional cortex during motor training led to significantly improved recovery compared to sham tDCS. It is important to underline that, to the best of our knowledge we are the first to apply tDCS *during* post stroke rehabilitation training in an animal model and test changes in behaviour using a sham-controlled, repeated measures design. Our results reached significance only when we excluded the non-grasping rats from the data analyses. However, based on the available data in healthy human volunteers, tDCS is only efficient in facilitating behavioural markers of learning when it is combined with executing a motor task (see discussion in chapter 3), which is in line with our results.

An important parameter that determines the effectiveness of post-stroke training and tDCS application is onset time. Although there is still no consensus on the optimal time window to begin the rehabilitation, it is known that an injured brain is susceptible to stimulation in the subacute phase (Kim et al., 2009). Yoon et al., (2012) reported that the application of tDCS in rats was more effective when started either 7 or 11 days post-stroke compared to 1 day post stroke, suggesting that anodal tDCS has no benefit when applied very early after the insult. However, it is also critical to not start the rehabilitation too late because early interventions in general have a more beneficial effect on long-term behavioural outcomes (Adkins & Jones, 2005; Biernaskie et al., 2004). In our study we started the concurrent application of tDCS with training 4 days after stroke onset. A significant difference between anodal and sham tDCS groups was observed after 6 training sessions (i.e. at day 9 post-stroke), it remains possible that anodal tDCS applied during the first 5 session of training contributed to this effect.

Current density is another important parameter that is likely to influence the effectiveness of non-invasive brain stimulation. There are very important methodologic differences between humans and

animal studies. The current density typically applied in animals (34.2 A/m^2) is 85 times higher compare to humans (0.4 A/m^2) (Brunoni et al., 2011). Although in our study we deliberately chose a lower current density (15.9 A/m^2) than previous studies (Notturno et al., 2014; Peruzzotti-Jametti et al., 2013; Yoon et al., 2012), it was still significantly higher compared to that typically applied to humans. Additionally, human studies use a symmetrical electrode sizes whereas in animal studies the reference electrode is almost 100 times larger than active electrode (Brunoni et al., 2011). These differences might be important for translational research, as previously shown from Mendonca et al., (2011) the tDCS effects are current distribution depended. For example, it could be that current applied in animals is more focal compare to humans. This might be due to highly asymmetrical electrodes in animals which increase the focality of the anodal electrode (Nitsche et al., 2007; Parazzini et al., 2011). However, the biggest difference is most likely the much higher current density applied in animal models compared to humans.

Only male rats (Sprague Dawley) were chosen for the behavioural experiment of our study in accordance with previous studies (Jiang et al., 2012; Kim et al., 2010; Yoon et al., 2012). The menstrual cycle in female rats might influence the outcome of the results.

We also observed substantial differences in the rat behaviour depending on the supplier. In a pilot study we obtained the same rat strain (Sprague-Dawley) from two different companies i.e. Harlan Company and Janvier Company. To our surprise, we noticed a difference in behavioural performance, even though they did not reach significance, main effect of *Group* F(1, 14) = 2.93; p = >0.05. There were differences in the time the rats needed to start grasping the pellets (see the first and the second training session on day 1) and even after 5 days of training there were differences in the overall amount of pellets grasped per training session, even though the data did not reach significance, *Time x Group* interaction F(3, 42) = 1.02; p > 0.05 (Fig 1). These differences might result from initial anxiety of the rats. We decided to order all animals from one company (Harlan Company). Nevertheless, even after taking this precaution we still noticed some differences amongst rats coming from the same company (delivery from Netherlands versus Italy of Harlan Company) but different breeding areas. These differences became clearly apparent when handling the rats and could result from the breeding or the delivery of the animals. For this reason we had a relatively long familiarization and pre-training period to ensure that rats exhibited relatively stable reaching behaviour prior to inducing the experimental stroke.

The results in the text are reported as mean (M), error bars in the figure display the standard error of the mean (SEM).
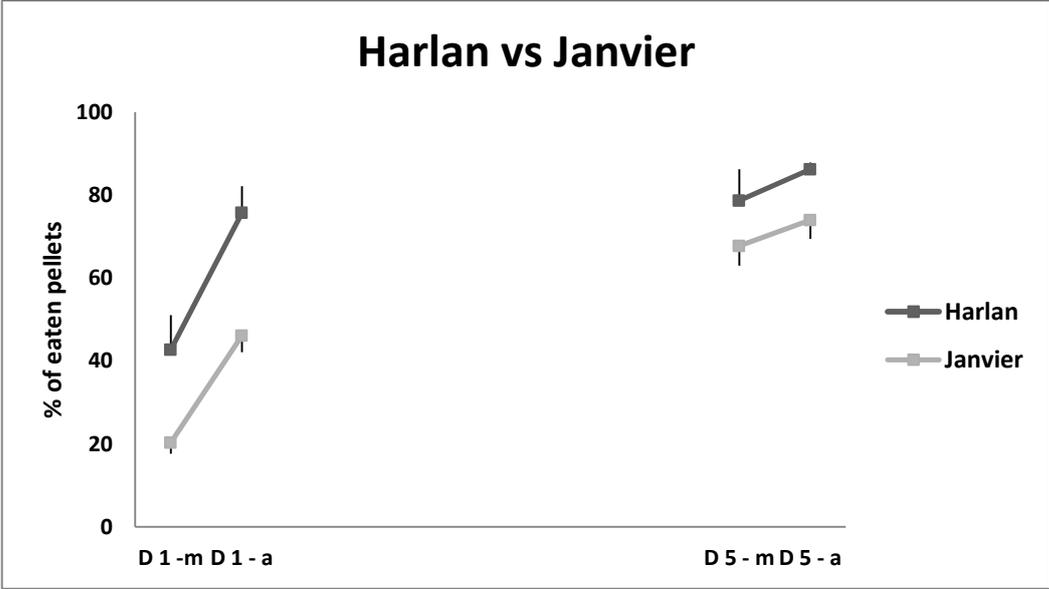
Figure 1 – The difference between Harlan Company (n = 12) and Janvier Company (n = 6). D1-m = day one morning, D1-a = day one afternoon (idem for D5-m and D5-a). Data shown as M ± SE.

Future suggestions

Our study showed that non-invasive brain stimulation techniques such as transcranial Direct Current Stimulation (tDCS) can facilitate long-term memory formation. It also showed some promising effects of tDCS for facilitating motor recovery in an animal model of stroke. However, further research will be needed to use this model and to gain a better understanding of the neural mechanisms underlying the effect of anodal tDCS stimulation. Furthermore, this model can be used to investigate which time point after the incident is optimal for applying tDCS and many other questions directly related to applying tDCS to the damaged brain. Therefore we suggest pursuing the use of tDCS for increasing plasticity after stroke for future research. The following aspects need further consideration: firstly, larger numbers of animals per subgroup (anodal/sham tDCS) are required to obtain better and more reliable results regarding the efficacy of tDCS intervention.

Secondly, although a large number of reaching tests/tasks has been developed for rats, the perfect test does not exist. Therefore, it is important to choose an appropriate behavioural test. Moreover, it might be important to include control groups that receive either anodal or sham tDCS during rest to show also for this rodent model that the strongest effects are revealed when anodal tDCS is combined with motor training. These tests are necessary to draw more reliable conclusions at the behavioural level which forms the basis for interpreting potential molecular aspects.

Third, the current density applied in humans is much lower when compared to rodents, mainly to minimize discomfort and damage of the skin. As current density is an important parameter that is likely to influence the effectiveness of tDCS, future studies should test a current density closer/comparable to the human studies which might be beneficial to better understand the effects of tDCS. However, if studies in the animal model would reveal that high stimulation intensities are necessary for facilitating recovery from stroke, one might consider minimally invasive procedures for placing the electrodes.

Another important point is to perform MRI examinations right after the experimental stroke to better match the lesion characteristics across groups.

Furthermore, to finally understand the underlying mechanism of how tDCS works, future research should analyse neural markers of plasticity. Currently, we developed an animal model which allows us to collect behavioural data but also to perform histology. Unfortunately, in the current study it was not possible to run molecular tests, i.e. immediate early genes, Western blot analyses following electrophysiology and immunohistochemistry tests but future research should take advantage of these techniques which might help/indicate to better understand why tDCS is beneficial for the plasticity and motor recovery.

Last but not least it is worth mentioning that anodal tDCS is not the only modulator of neuroplasticity and learning and that it might be interesting to further pursue behavioural manipulation as the reward and punishment scheme used here. In future experiments a larger number of participants (rewarded/punished) is required in order to draw a more reliable conclusion, and it would be advisable to use a different task. Furthermore, a control group should be included which might help to properly interpret the differences amongst the conditions. The control group should only receive motivationally neutral feedback irrespective of performance. However, the subjects in the control group should receive a similar/comparable amount of money as the other two groups (rewarded or punished).

Conclusion

In summary, we showed that monetary reward or punishment had little influence on use-dependent plasticity triggered by motor practice which might result from the nature of the task used in this PhD thesis. By contrast, tDCS modulated long-term use-dependent plasticity caused by repetitive training in healthy humans and using an animal model we could show that the application of tDCS during early post-stroke training is beneficial in an animal model of stroke. Our results suggest that tDCS is a promising adjuvant to facilitate motor recovery following stroke. However, while the effects of tDCS look very promising, further research is needed because the underlying mechanisms are not well-understood. Animal models that closely mimic tDCS application in human patients, as developed here, can be used for gaining a better understanding of the underlying mechanisms.

References

Adkins, D. L., & Jones, T. a. (2005). D-Amphetamine enhances skilled reaching after ischemic cortical lesions in rats. *Neuroscience Letters*, *380*, 214–218. http://doi.org/10.1016/j.neulet.2005.01.036

Ballermann, M., Metz, G. a S., McKenna, J. E., Klassen, F., & Whishaw, I. Q. (2001). The pasta matrix reaching task: A simple test for measuring skilled reaching distance, direction, and dexterity in rats. *Journal of Neuroscience Methods*, *106*, 39–45. http://doi.org/10.1016/S0165-0270(01)00326-0

Bhakta, B. B., Hartley, S., Holloway, I., Couzens, J. A., Ford, G. A., Meads, D., … Farrin, A. J. (2014). The DARS (Dopamine Augmented Rehabilitation in Stroke) trial : protocol for a randomised controlled trial of Co-careldopa treatment in addition to routine NHS occupational and physical therapy after stroke, 1–11.

Biernaskie, J., Chernenko, G., & Corbett, D. (2004). Efficacy of rehabilitative experience declines with time after focal ischemic brain injury. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *24*(5), 1245–1254. http://doi.org/10.1523/JNEUROSCI.3834-03.2004

Boggio, P. S., Castro, L. O., Savagim, E. a., Braite, R., Cruz, V. C., Rocha, R. R., … Fregni, F. (2006). Enhancement of non-dominant hand motor function by anodal transcranial direct current stimulation. *Neuroscience Letters*, *404*, 232–236. http://doi.org/10.1016/j.neulet.2006.05.051

Brunoni, A. R., Fregni, F., & Pagano, R. L. (2011). Translational research in transcranial direct current stimulation (tDCS): A systematic review of studies in animals. *Reviews in the Neurosciences*, *22*(4), 471–481. http://doi.org/10.1515/RNS.2011.042

Buma, F., Kwakkel, G., & Ramsey, N. (2013). Understanding upper limb recovery after stroke. *Restorative Neurology and Neuroscience*, *31*(6), 707–722. http://doi.org/10.3233/RNN-130332

Bütefisch, C. M., Davis, B. C., Wise, S. P., Sawaki, L., Kopylev, L., Classen, J., … Bu, C. M. (2000). Mechanisms of use-dependent plasticity in the human motor cortex. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(7), 3661–5. http://doi.org/10.1073/pnas.050350297

Classen, J., Liepert, J., Wise, S. P., Hallett, M., & Cohen, L. G. (1998). Rapid plasticity of human cortical movement representation induced by practice. *Journal of Neurophysiology*, *79*, 1117–1123. http://doi.org/citeulike-article-id:2143137

Datta, A., Truong, D., Minhas, P., Parra, L. C., & Bikson, M. (2012). Inter-individual variation during transcranial direct current stimulation and normalization of dose using MRI-derived computational models. *Frontiers in Psychiatry*, *3*(October), 1–8. http://doi.org/10.3389/fpsyt.2012.00091

Daw, N. D., Kakade, S., & Dayan, P. (2002). Opponent interactions between serotonin and dopamine. *Neural Networks*, *15*(4-6), 603–616. http://doi.org/10.1016/S0893-6080(02)00052-7

Dietrich, W. D., Ginsberg, M. D., Busto, R., & Watson, B. D. (1986). Photochemically induced cortical infarction in the rat. 2. Acute and subacute alterations in local glucose utilization. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *6*, 195–202. http://doi.org/10.1038/jcbfm.1986.32

Fritsch, B., Reis, J., Martinowich, K., Schambra, H. M., Ji, Y., Cohen, L. G., & Lu, B. (2011). Direct current stimulation promotes BDNF-dependent synaptic plasticity: Potential implications for motor learning. *NIH Public Access*, *66*(2), 198–204. http://doi.org/10.1016/j.neuron.2010.03.035.Direct

Galea, J. M., Mallia, E., Rothwell, J., & Diedrichsen, J. (2015). The dissociable effects of punishment and reward on motor learning. *Nature Neuroscience*, *7*(February). http://doi.org/10.1038/nn.3956

Hadipour-Niktarash, A., Lee, C. K., Desmond, J. E., & Shadmehr, R. (2007). Impairment of retention but not acquisition of a visuomotor skill through time-dependent disruption of primary motor cortex. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(49), 13413–13419. http://doi.org/10.1523/JNEUROSCI.2570-07.2007

Hesse, S., Werner, C., Schonhardt, E. M., Bardeleben, A., Jenrich, W., & Kirker, S. G. B. (2007). Combined transcranial direct current stimulation and robot-assisted arm training in subacute stroke patients: a pilot study. *Restorative Neurology and Neuroscience*, *25*, 9–15. http://doi.org/10.1177/1545968311413906

Hosp, J. a., & Luft, A. R. (2013). Dopaminergic meso-cortical projections to M1: Role in motor learning and motor cortex plasticity. *Frontiers in Neurology*, *4 OCT*(October), 1–7. http://doi.org/10.3389/fneur.2013.00145

Jiang, T., Xu, R. X., Zhang, a. W., Di, W., Xiao, Z. J., Miao, J. Y., … Fang, Y. N. (2012). Effects of transcranial direct current stimulation on hemichannel pannexin-1 and neural plasticity in rat model of cerebral infarction. *Neuroscience*, *226*, 421–426. http://doi.org/10.1016/j.neuroscience.2012.09.035

Kantak, S. S., Mummidisetty, C. K., & Stinear, J. W. (2012). Primary motor and premotor cortex in implicit sequence learning - Evidence for competition between implicit and explicit human motor memory systems. *European Journal of Neuroscience*, *36*(February), 2710–2715. http://doi.org/10.1111/j.1460-9568.2012.08175.x

Kerr, A. L., & Tennant, K. a. (2014). Compensatory Limb Use and Behavioral Assessment of Motor Skill Learning Following Sensorimotor Cortex Injury in a Mouse Model of Ischemic Stroke. *Journal of Visualized Experiments*, (July), 1–7. http://doi.org/10.3791/51602

Kim, D. Y., Ohn, S. H., Yang, E. J., Park, C.-I., & Jung, K. J. (2009). Enhancing motor performance by anodal transcranial direct current stimulation in subacute stroke patients. *American Journal of Physical Medicine & Rehabilitation / Association of Academic Physiatrists*, *88*, 829–836. http://doi.org/10.1097/PHM.0b013e3181b811e3

Kim, S. J., Kim, B. K., Ko, Y. J., Bang, M. S., Kim, M. H., & Han, T. R. (2010). Functional and histologic changes after repeated transcranial direct current stimulation in rat stroke model. *Journal of Korean Medical Science*, *25*, 1499–1505. http://doi.org/10.3346/jkms.2010.25.10.1499

Krakauer, J. W., & Mazzoni, P. (2011). Human sensorimotor learning: Adaptation, skill, and beyond. *Current Opinion in Neurobiology*, *21*(4), 636–644. http://doi.org/10.1016/j.conb.2011.06.012

Labat-gest, V., & Tomasi, S. (2013). Photothrombotic ischemia: a minimally invasive and reproducible photochemical cortical lesion model for mouse stroke studies. *Journal of Visualized Experiments : JoVE*, (76), 1–6. http://doi.org/10.3791/50370

Li, L. M., Uehara, K., & Hanakawa, T. (2015). The contribution of interindividual factors to variability of response in transcranial direct current stimulation studies. *Frontiers in Cellular Neuroscience*, *9*(May). http://doi.org/10.3389/fncel.2015.00181

Lindau, N. T., Bänninger, B. J., Gullo, M., Good, N. a., Bachmann, L. C., Starkey, M. L., & Schwab, M. E. (2014). Rewiring of the corticospinal tract in the adult rat after unilateral stroke and anti-Nogo-A therapy. *Brain*, *137*(3), 739–756. http://doi.org/10.1093/brain/awt336

Mendonca, M. E., Santana, M. B., Baptista, A. F., Datta, A., Bikson, M., Fregni, F., & Araujo, C. P. (2011). Transcranial DC stimulation in fibromyalgia: Optimized cortical target supported by high-resolution computational models. *Journal of Pain*, *12*(5), 610–617. http://doi.org/10.1016/j.jpain.2010.12.015

Miranda, P. C., Lomarev, M., & Hallett, M. (2006). Modeling the current distribution during transcranial direct current stimulation. *Clinical Neurophysiology*, *117*, 1623–1629.

http://doi.org/10.1016/j.clinph.2006.04.009

Missitzi, J., Gentner, R., Misitzi, A., Geladas, N., Politis, P., Klissouras, V., & Classen, J. (2013). Heritability of motor control and motor learning. *Physiological Reports*, *1*, e00188. http://doi.org/10.1002/phy2.188

Murphy, T. H., & Corbett, D. (2009). Plasticity during stroke recovery: from synapse to behaviour. *Nature Reviews. Neuroscience*, *10*(DECEmbEr), 861–872. http://doi.org/10.1038/nrn2735

Nitsche, M. a, Doemkes, S., Karaköse, T., Antal, a, Liebetanz, D., Lang, N., … Paulus, W. (2007). Shaping the effects of transcranial direct current stimulation of the human motor cortex. *Journal of Neurophysiology*, *97*(4), 3109–3117. http://doi.org/10.1152/jn.01312.2006

Notturno, F., Pace, M., Zappasodi, F., Cam, E., Bassetti, C. L., & Uncini, A. (2014). Neuroprotective effect of cathodal transcranial direct current stimulation in a rat stroke model. *Journal of the Neurological Sciences*, *342*(1-2), 146–151. http://doi.org/10.1016/j.jns.2014.05.017

Nudo, R. J. (2013). Recovery after brain injury: mechanisms and principles. *Frontiers in Human Neuroscience*, *7*(December), 887. http://doi.org/10.3389/fnhum.2013.00887

Opitz, A., Paulus, W., Will, A., & Thielscher, A. (2015). Anatomical determinants of the electric field during transcranial direct current stimulation. *NeuroImage*, *109*, 2. http://doi.org/10.1016/j.neuroimage.2015.01.033

Orban de Xivry, J.-J., & Shadmehr, R. (2014). Electrifying the motor engram: effects of tDCS on motor learning and control. *Experimental Brain Research*, *232*, 3379–3395. http://doi.org/10.1007/s00221-014-4087-6

Parazzini, M., Fiocchi, S., Rossi, E., Paglialonga, a, & Ravazzani, P. (2011). Transcranial Direct Current Stimulation: Estimation of the Electric Field and of the Current Density in an Anatomical Human Head Model. *IEEE Transactions on Biomedical Engineering*, *58*(6), 1773–1780. http://doi.org/10.1109/TBME.2011.2116019

Peruzzotti-Jametti, L., Cambiaghi, M., Bacigaluppi, M., Gallizioli, M., Gaude, E., Mari, S., … Leocani, L. (2013). Safety and efficacy of transcranial direct current stimulation in acute experimental ischemic stroke. *Stroke*, *44*, 3166–3174. http://doi.org/10.1161/STROKEAHA.113.001687

Picelli, A., Chemello, E., Castellazzi, P., Roncari, L., Waldner, A., Saltuari, L., & Smania, N. (2015). Combined effects of transcranial direct current stimulation (tDCS) and transcutaneous spinal direct current stimulation (tsDCS) on robot - assisted gait training in patients with chronic stroke: A pilot, double blind, randomized controlled trial. *Restor Neurol Neurosci*, 357–68.

Reis, J., Fischer, J. T., Prichard, G., Weiller, C., Cohen, L. G., & Fritsch, B. (2013). Time- but Not Sleep-Dependent Consolidation of tDCS-Enhanced Visuomotor Skills. *Cerebral Cortex (New York, N.Y. : 1991)*, 1–9. http://doi.org/10.1093/cercor/bht208

Rioult-Pedotti, M. S., Friedman, D., & Donoghue, J. P. (2000). Learning-induced LTP in neocortex. *Science (New York, N.Y.)*, *290*(2000), 533–536. http://doi.org/10.1126/science.290.5491.533

Rioult-Pedotti, M. S., Friedman, D., Hess, G., & Donoghue, J. P. (1998). Strengthening of horizontal cortical connections following skill learning. *Nature Neuroscience*, *1*(3), 230–234. http://doi.org/10.1038/678

Rosenkranz, K., Kacar, A., & Rothwell, J. C. (2007). Differential modulation of motor cortical plasticity and excitability in early and late phases of human motor learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *27*(44), 12058–12066. http://doi.org/10.1523/JNEUROSCI.2663-07.2007

Rossi, C., Sallustio, F., Di Legge, S., Stanzione, P., & Koch, G. (2013). Transcranial direct current stimulation of the affected hemisphere does not accelerate recovery of acute stroke patients. *European Journal of Neurology : The Official Journal of the European Federation of Neurological Societies*, *20*, 202–4. http://doi.org/10.1111/j.1468-1331.2012.03703.x

Sattler, V., Acket, B., Raposo, N., Albucher, J.-F., Thalamas, C., Loubinoux, I., … Simonetta-Moreau, M. (2015). Anodal tDCS Combined With Radial Nerve Stimulation Promotes Hand Motor Recovery in the Acute Phase After Ischemic Stroke. *Neurorehabilitation and Neural Repair*. http://doi.org/10.1177/1545968314565465

Saucedo Marquez, C. M., Zhang, X., Swinnen, S. P., Meesen, R., & Wenderoth, N. (2013). Task-specific effect of transcranial direct current stimulation on motor learning. *Frontiers in Human Neuroscience*, *7*(July), 333. http://doi.org/10.3389/fnhum.2013.00333

Schmidt, A., Hoppen, M., Strecker, J.-K., Diederich, K., Schäbitz, W.-R., Schilling, M., & Minnerup, J. (2012). Photochemically induced ischemic stroke in rats. *Experimental & Translational Stroke Medicine*, *4*, 13. http://doi.org/10.1186/2040-7378-4-13

Stagg, C. J., Jayaram, G., Pastor, D., Kincses, Z. T., Matthews, P. M., & Johansen-Berg, H. (2011). Polarity and timing-dependent effects of transcranial direct current stimulation in explicit motor learning. *Neuropsychologia*, *49*(5), 800–804. http://doi.org/10.1016/j.neuropsychologia.2011.02.009

Tamura, a, Graham, D. I., McCulloch, J., & Teasdale, G. M. (1981). Focal cerebral ischaemia in the rat:

1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *Journal of Cerebral Blood Flow and Metabolism : Official Journal of the International Society of Cerebral Blood Flow and Metabolism*, *1*, 53–60. http://doi.org/10.1038/jcbfm.1981.6

Tanaka, S., Hanakawa, T., Honda, M., & Watanabe, K. (2009). Enhancement of pinch force in the lower leg by anodal transcranial direct current stimulation. *Experimental Brain Research*, *196*, 459–465. http://doi.org/10.1007/s00221-009-1863-9

Tecchio, F., Zappasodi, F., Assenza, G., Tombini, M., Vollaro, S., Barbati, G., & Rossini, P. M. (2010). Anodal transcranial direct current stimulation enhances procedural consolidation. *Journal of Neurophysiology*, *104*(June 2010), 1134–1140. http://doi.org/10.1152/jn.00661.2009

Teo, J. T. H., Bentley, G., Lawrence, P., Soltesz, F., Miller, S., Willé, D., … Nathan, P. J. (2014). Late cortical plasticity in motor and auditory cortex: role of met-allele in BDNF Val66Met polymorphism. *The International Journal of Neuropsychopharmacology / Official Scientific Journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)*, *17*(5), 705–13. http://doi.org/10.1017/S1461145713001636

Wächter, T., Lungu, O. V, Liu, T., Willingham, D. T., & Ashe, J. (2009). Differential effect of reward and punishment on procedural learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *29*(2), 436–443. http://doi.org/10.1523/JNEUROSCI.4132-08.2009

Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S., & Ginsberg, M. D. (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Annals of Neurology*. http://doi.org/10.1002/ana.410170513

Whishaw, I. Q. (2000). Loss of the innate cortical engram for action patterns used in skilled reaching and the development of behavioral compensation following motor cortex lesions in the rat. *Neuropharmacology*, *39*(5), 788–805. http://doi.org/10.1016/S0028-3908(99)00259-2

Yoon, K. J., Oh, B. M., & Kim, D. Y. (2012). Functional improvement and neuroplastic effects of anodal transcranial direct current stimulation (tDCS) delivered 1 day vs. 1 week after cerebral ischemia in rats. *Brain Research*, *1452*, 61–72. http://doi.org/10.1016/j.brainres.2012.02.062

Appositions

1 –   The prevalence of several neurological disorders such as headache, epilepsy, dementia, Parkinson's disease, multiple sclerosis, stroke and cerebral palsy, tends to be higher in Albania than in other countries. Differences may be explained by study design, population structure, and/or genetic and environmental factors however, further research needs to be done to identify these factors prior to the introduction of preventive strategies.

2 –   Multicultural exchange opens the mind and helps to change the students' view on different ethnic groups.

3 –   Studying demands a lot of time and energy, therefore extracurricular activities are very important to maintain a healthy, productive, and balanced life.

Curriculum Vitae

Orjon Rroji was born in Shkoder, Albania on September 28th, 1978. He obtained his bachelor degree in General Biology at University of Padua and a master of science in Biology of Behaviour at University of Florence in Italy. In 2010 he started a PhD under supervision of Prof. Dr. Nicole Wenderoth and Prof. Dr. Bart Nuttin at the Research Centre for Movement Control and Neuroplasticity at the KU Leuven.

List of publications

Articles

Rroji O, van Kuyck K, Nuttin B, Wenderoth N. Anodal tDCS over the Primary Motor Cortex Facilitates Long-Term Memory Formation Reflecting Use-Dependent Plasticity. PLoS One. 2015 May 21;10(5):e0127270. doi: 10.1371/journal.pone.0127270. eCollection 2015

Abstracts and Posters presentations

Rroji O., Leenus D., van Kuyck K., Nuttin B., Hellings N., Meesen R., Swinnen S., Wenderoth N. "Interhemispheric transfer of motor skill tested in a rat model". Poster presented at the annual Meeting of Society for Neuroscience, 13 – 17 October 2012 in New Orleans, USA

Rroji O., Hebbelinck A., Wynants K., Ceulemans E., Wenderoth N. "Reward modulates changes of corticomotor excitability by motor skills". Poster presented at 8th FENS forum of Neuroscience conference, 14 – 18 July 2012 Barcelona, Spain

Rroji O., Bekkers E., Pauwels L., Wenderoth N. "Does tDCS over the primary motor cortex have an effect on the consolidation of motor memories". Poster presented at ECSS 17th annual Congress, 4 – 7 July 2012 Brugges, Belgium

Pauwels L., Bekkers E., **Rroji O.,** Wenderoth N. "Does non-invasive brain stimulation over the primary cortex improve consolidation of motor memories?" Poster presented at Vereniging voor Kinesiologie symposium "Beweging doorheen de jaren", 16 December 2011 Universiteit Gent, Belgium

Rroji O., Bekkers E., Pauwels L., Wenderoth N. "Does tDCS over the M1 improve consolidation of motor memories". Poster presented at Magstim TMS Summer School 28 – 29 May 2011 Oxford, UK

Acknowledgement

Firstly, my heartily thanks go to my supervisor and mentor, Nici Wenderoth, who showed me how and what does it mean to do research, and who advice, taught and support me during all these years. Another heartily thanks goes to my co-supervisor Prof. Bart Nuttin who wisely advice and supported me during all these years. Of course another special thank is for my co-supervisor Kris van Kuyck, for supervising me and for being my friend. A special thank go to the jury members Prof. Johan Lefevre, Prof. Hilde Feys, Prof. Detlef Balschun, Prof. Ralf Krampe, Prof. Romain Meeusen for their advice.

I also wish to thank my lab mates and my colleagues, in both offices and labs where I have worked, for their friendship and collaboration. My thanks go also to my friends everywhere they are in Belgium, Italy, Germany, Albania, Australia and all around the world for their friendship and support.

Finally, I wish to thanks my parents, who always showed me that they are proud of me, and I am very proud to be their son.

ANYWAY – Mother Teresa

People are unreasonable, illogical, and self-centered,

LOVE THEM ANYWAY

If you do good, people will accuse you of selfish, ulterior motives,

DO GOOD ANYWAY

If you are successful, you win false friends and true enemies,

SUCCEED ANYWAY

The good you do will be forgotten tomorrow,

DO GOOD ANYWAY

Honesty and frankness make you vulnerable,

BE HONEST AND FRANK ANYWAY

What you spent years building may be destroyed overnight,

BUILD ANYWAY

People really need help but may attack you if you help them,

HELP PEOPLE ANYWAY

Give the world the best you have and you'll get kicked in the teeth,

GIVE THE WORLD THE BEST YOU'VE GOT ANYWAY