MODULATION OF NEUROPLASTICITY IN HUMANS BY ADVANCED STIMULATION PROTOCOLS AND NEUROMODULATORS

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Statement of Originality

I hereby declare that this thesis has been written independently with no other sources and aids than quoted in the text.

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Abbreviations

5-HT	5-hydroxytryptamine/serotonin
ADM	abductor digiti minimi
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CSP	cortical silent period
FDI	first dorsal interosseus
GABA	γ-aminobutyric acid
G-protein	guanosine nucleotide-binding protein
ICF	intracortical facilitation
I-O curve	input-output curve
ISI	interstimulus interval
LTD	long term depression
LTP	long term potentiation
M1	primary motor cortex
mAChR	muscarinic acetylcholine receptor
MEP	motor evoked potential
MRI	magnetic resonance imaging
MT	motor threshold
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-D-aspartate
PAS	paired associative stimulation
PAS10	paired associative stimulation with 10ms interstimulus interval
PAS25	paired associative stimulation with 25ms interstimulus interval
rTMS	repetitive transcranial magnetic stimulation
SICI	short-latency intracortical inhibition
SSRI	selective serotonin reuptake inhibitor
STDP	spike-timing dependent plasticity
tDCS	transcranial direct current stimulation
TES	transcranial electric stimulation
TMS	transcranial magnetic stimulation
tRNS	transcranial random noise stimulation

Chapter 1 - Introduction

Neuroplasticity is a feature of the human brain to dynamically reorganize itself structurally as well as functionally in response to changes of the environment, behavior or brain injury. It can be accomplished via adding, removing, strengthening or weakening of synaptic connections as well as neurogenesis (Pascual-Leone et al., 2005, Pascual-Leone et al., 2011). Besides being one of the most important physiological mechanisms of learning, memory and other cognitive processes, pathologically altered neuroplasticity can cause neuropsychiatric diseases. The discovery and development of non-invasive brain stimulation techniques in the last decades has given researchers the opportunity to study neuroplasticity in humans. Transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS) are widely used techniques for non-invasively inducing and monitoring these processes in the human brain (Nitsche and Paulus, 2000, Stefan et al., 2000, Nitsche et al., 2008, Ziemann et al., 2008).

The present work is divided into two parts: first, the deeper exploration of mechanisms influencing brain plasticity using modified brain stimulation protocols and the second part, representing the impact of two major neuromodulators (serotonin and nicotine) on non-invasive brain stimulation-induced neuroplasticity. Several studies have previously demonstrated the impact of different neuromodulators on different types of plasticity in humans (Kuo et al., 2007, Kuo et al., 2008, Monte-Silva et al., 2009, Nitsche et al., 2009, Monte-Silva et al., 2010b, Thirugnanasambandam et al., 2012). In this thesis we aimed to study the impact of serotonin on synapse-specific focal plasticity induced by PAS and the dose-dependent effect of $\alpha_4\beta_2$ nicotinic receptor activation on plasticity.

The first chapter of this thesis will introduce basic information about neuroplasticity, neuromodulatory systems and techniques used in the studies presented in the second chapter. The last chapter will summarize the findings of the presented studies and offer an outlook and possible future research directions in the field.

1.1. Plasticity in the central nervous system

Neuroplasticity is an intrinsic property of the nervous system to modify, optimize and reorganize itself structurally or functionally in response to physiological or environmental changes and injuries (Citri and Malenka, 2008). Functional plasticity accomplished by long-lasting changes in the central nervous system, such as long term potentiation (LTP) and long term depression (LTD), is considered to be a mechanism of learning and memory formation. LTP and LTD have been most frequently studied for glutamatergic synapses in various brain areas and have been shown to be mediated by NMDA receptors that have calcium channel properties (Bliss and Collingridge, 1993, Malenka and Bear, 2004). Therefore, the major factor determining the direction of plasticity at a specific synapse is the postsynaptic calcium concentration (Lisman, 2001). It has been shown that low postsynaptic calcium concentration results in LTD, high concentration in LTP, and at a medium concentration a so-called “no man’s land” exists at which no plasticity results (Cho et al., 2001, Lisman, 2001). Very high Ca^{2+} concentrations can also result in no plasticity due to activation of hyperpolarizing potassium channels (Misonou et al., 2004). Low intracellular calcium concentration triggers a cascade of intracellular reactions leading to removal of AMPA receptors from the synaptic membrane, weakening synaptic strength and resulting in LTD. In contrast, high calcium influx into the neuron results in activation of the opposite mechanism, enhancing the insertion of AMPA receptors in the subsynaptic membrane, resulting in LTP (Cummings et al., 1996, Malenka and Bear, 2004).

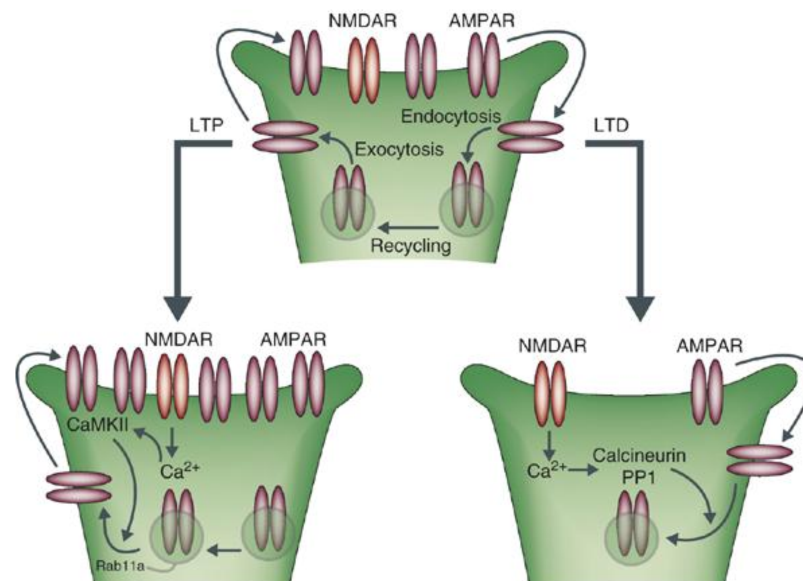

Figure 1. Illustration of LTP and LTD induction mechanisms at a glutamatergic synapse. Depending on intracellular calcium concentration, a specific cascade of cytoplasmic reactions is triggered, leading to either LTP or LTD. Induction of LTP is followed by an addition of AMPA receptors to the synaptic membrane via exocytosis, respectively, removal of AMPA receptors occurs after LTD induction via endocytosis, thus the strength and efficacy of synaptic transmission is altered (adapted from (Citri and Malenka, 2008)).

1.2. Neuroplasticity in humans: the motor cortex as a model

Neuroplasticity in humans has been the subject of intensive studies during the past decades, and has been increasingly recognized as an important physiological basis for learning, and memory processes. Various studies demonstrate brain plasticity in healthy individuals. For example, in mathematicians gray matter density in the left inferior frontal and bilateral inferior parietal lobules (regions, related to mathematical thinking) is significantly higher than in controls (Aydin et al., 2007). Similarly, magnetic resonance imaging (MRI) studies have revealed increased gray matter density in motor, auditory cortex and cerebellum in musicians, compared to controls (Gaser and Schlaug, 2003). Another MRI study revealed increased gray matter density in the left inferior parietal cortex of bilingual subjects compared to monolinguals (Mechelli et al., 2004). Changes in gray matter density in regions associated with learning and memory (posterior and lateral parietal cortex and hippocampus) were demonstrated in medical students who were preparing for an exam, compared to controls who did not study at that time (Draganski et al., 2006). Studies have also revealed that blind subjects have larger representation of fingers in the somatosensory maps due to increased tactile discrimination abilities (Pascual-Leone and Torres, 1993) and rearrangements in visual areas due to echolocation (Thaler et al., 2011).

In the clinical domain, studies suggest that depression might be caused by altered brain plasticity, namely, enhanced inhibitory and reduced excitatory plasticity (Christoffel et al., 2011). In accordance, results of a recently published study revealed deficits in motor learning and PAS25-induced excitatory plasticity in patients with depression compared to healthy controls (Player et al., 2013). Imaging studies also show changes in hippocampal volume in patients

suffering from depression (Sheline et al., 1996, Sheline et al., 2003, Campbell et al., 2004). In stroke patients, excitatory plasticity was also shown to be reduced (Traversa et al., 1997, Traversa et al., 1998). Functional MRI studies in stroke patients subjects also revealed changes in motor and sensory maps throughout the rehabilitation process, correlating with recovery (Liepert et al., 1998, Levy et al., 2001, Hodics et al., 2006, Johansen-Berg et al., 2010).

In recent years, non-invasive brain stimulation techniques, such as repetitive transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS) allow researchers to induce plasticity in humans (Nitsche and Paulus, 2000, Stefan et al., 2000, Huang et al., 2004a). These techniques induce changes of cortical excitability and can be monitored by recording TMS-elicited motor evoked potentials (MEPs). The motor cortex was used as a model in most of the studies conducted so far, as it is relatively well explored, easy reachable using TMS and tDCS, because it is situated at the brain surface (especially, small hand muscle representations), and MEPs are relatively objective output parameters for measuring cortical excitability. In all our studies, we obtained MEPs from the abductor digiti minimi (ADM) or first dorsal interosseus (FDI) muscles elicited by single or paired-pulse TMS to monitor plasticity.

1.3. Non-invasive brain stimulation techniques

The first method to non-invasively access and stimulate cortical neurons in the human brain was transcranial electric stimulation (TES) (Merton and Morton, 1980). To activate cortical neurons and induce action potentials, this method uses a high voltage current, which also activates cutaneous and meningeal pain receptors as well as head muscles, and therefore is uncomfortable and painful for the subjects. In 1985 another non-invasive brain stimulation technique – TMS was developed (Barker et al., 1985). Unlike TES, the TMS magnetic pulse penetrates the skull, induces a secondary electric field in the brain and neuronal action potentials, without activating pain receptors and head muscles. Thus, TMS became very popular for monitoring of cortical excitability. Single-pulse TMS-induced MEPs have shown to be objective measures of cortical excitability (Rothwell, 1993). Repetitively applied TMS pulses have

been shown to induce long-lasting cortical excitability changes depending on the frequency of application (Pascual-Leone and Hallett, 1994, Huang et al., 2004a). When combined with peripheral nerve stimulation, TMS can also produce excitability changes, depending on the interstimulus interval (ISI). This method is called paired associative stimulation (PAS) and induces plasticity similar to spike-timing dependent plasticity (STDP), which is thought to be involved in learning and memory processes (Stefan et al., 2002, Wolters et al., 2003, Caporale and Dan, 2008).

Apart from rTMS, another non-invasive brain stimulation technique was introduced some years ago, which induces polarity-dependent changes in cortical excitability using subthreshold direct current (tDCS) (Nitsche and Paulus, 2000).

1.3.1. Transcranial magnetic stimulation

TMS pulses induce rapidly changing magnetic fields in cortical structures, which results in secondary electric fields and current flow opposite to magnetic coil orientation. If this current is sufficiently large, it can depolarize neurons. In the motor cortex, a suprathreshold TMS pulse can activate cortical representations of a specific hand or leg muscle, eliciting motor evoked potentials (MEP). TMS-elicited MEPs can be recorded using surface electromyography (EMG) electrodes (Rothwell, 1993). Single pulse MEPs are used in our studies to precisely monitor changes in cortical excitability before and after pharmacological and/or non-invasive brain stimulation interventions. Apart from single-pulse TMS, we also used other single and paired pulse TMS protocols to explore various parameters of intracortical and corticospinal excitability, such as, active and resting motor thresholds (MTs), input-output (I-O) curves, I-waves, short latency intracortical inhibition (SICI), intracortical facilitation (ICF), and cortical silent period (CSP) (Fuhr et al., 1991, Kujirai et al., 1993, Ziemann and Rothwell, 2000, Abbruzzese and Trompetto, 2002).

In our studies, TMS was also used as plasticity-inducing protocol combined with peripheral nerve stimulation (see section 1.2.3).

1.3.2. Transcranial direct current stimulation

Transcranial direct current stimulation is a non-invasive brain stimulation technique that can induce long lasting changes in cortical excitability. Current applied during tDCS is subthreshold, therefore unable to elicit action potentials (Nitsche and Paulus, 2000). The induced weak electric current penetrates through the skull and affects neuronal populations under the stimulation electrodes by shifting their resting membrane potential to the direction of de- or hyperpolarization, therefore making them more or less likely to be excited. These excitability changes depend on electrode polarity and can outlast the stimulation duration. Anodal stimulation induces depolarization and higher excitability, whereas cathodal tDCS has the opposite, hyperpolarizing effect (Nitsche and Paulus, 2001, Nitsche et al., 2003b, Nitsche et al., 2008), when applied within the limits of standard protocols. Similar polarity-dependent long-lasting effects have been shown before in slice and animal experiments (Bindman et al., 1964, Purpura and McMurtry, 1965).

Pharmacological studies show that tDCS after-effects are NMDA receptor- and calcium-dependent (Nitsche et al., 2003a). Administration of NMDA receptor antagonists or Ca^{2+} channel blockers abolish tDCS-induced plasticity (Liebetanz et al., 2002, Nitsche et al., 2003a), indicating that tDCS after-effects share similarities with LTD and LTP induction mechanisms in animal studies (Lisman, 2001), and alter the strength of glutamatergic synapses.

1mA tDCS has been widely used in research as well as clinical studies. The current intensity and duration has been increased in numerous more recently conducted studies, based on the assumption that this will result in desired longer/stronger stimulation after-effects. Although several studies demonstrated clinical or cognitive effects of 2mA tDCS (Fregni et al., 2006a, Fregni et al., 2006b, Brunoni et al., 2011, Bueno et al., 2011, Brunelin et al., 2012), its impact on cortical excitability has not yet been explored physiologically.

In all our studies, direct current was applied through pairs of saline-soaked surface sponge electrodes and delivered by a battery-driven constant current stimulator. One electrode was fixed over the motor cortex (the area representing the FDI or ADM muscle, as identified by TMS) and the return electrode was fixed contralaterally, over the right supraorbital area. The current

intensity was 1 or 2mA, applied for 9 (1mA cathodal tDCS), 13 (1mA anodal tDCS) or 20 minutes (2mA cathodal/anodal tDCS, 1mA cathodal tDCS) in the different studies, inducing after-effects lasting for about one hour after stimulation end.

1.3.3. Paired associative stimulation

Paired associative stimulation is a technique which combines a TMS pulse with low-frequency electric suprathreshold peripheral nerve stimulation, inducing neuroplastic changes, similar to spike-timing dependent plasticity (STDP). STDP is thought to be the underlying mechanism of learning/memory processes (Caporale and Dan, 2008). The direction of PAS-induced cortical excitability changes depends on the interstimulus interval between peripheral and TMS pulses. The peripheral stimulus is applied first and is followed by the TMS pulse. If the TMS pulse is applied 20-25ms after the peripheral stimulus (approximately the time the latter reaches M1), synchronous activation of the motor neurons occurs through somatosensory and motor cortical connections and facilitatory plasticity is induced. In contrast, when the TMS pulse is applied less than 20ms after the peripheral stimulus, it precedes the arrival of the peripheral pulse, therefore asynchronous activation of the above mentioned connections results in inhibitory plasticity (Stefan et al., 2000, Stefan et al., 2002, Wolters et al., 2003).

PAS after-effects share some characteristics with those of tDCS, as they are also NMDA receptor and calcium dependent (Stefan et al., 2002, Wolters et al., 2003) and therefore thought to be LTP- and LTD-like. Unlike tDCS, which affects a big population of neurons under relatively large stimulation electrodes, PAS is thought to be focal and synapse-specific, affecting only small, specific population of neurons.

In our experiments, the peripheral electric pulse was delivered over the right ulnar nerve at the level of the wrist at an intensity of 300% of the sensory perceptual threshold, followed by a TMS pulse over the M1 representation of the abductor digiti minimi muscle at ISIs of 10ms (PAS10) or 25ms (PAS25) at a frequency of 0.05Hz. After PAS10, the asynchronous arrival of two pulses induced inhibitory plasticity, while after PAS25 their synchronous arrival to the motor cortex resulted in facilitatory plasticity (Stefan et al., 2002, Wolters et al., 2003).

1.4. Neuromodulators

Neuromodulators are a class of neurotransmitters with specific features. Depending on postsynaptic receptor composition, cortical background activity, and dosage, amongst other factors, they can elicit either excitatory or inhibitory actions on cortical neurons and also modulate the release of other neurotransmitters (serotonin, dopamine, acetylcholine, etc). Recent studies suggest that synaptic plasticity does not always depend only on the pre-and postsynaptic neuronal activity, but also on the presence of neuromodulators (Malenka and Bear, 2004). Unlike classical chemical synapses, where the presynaptic neuron directly affects the target cell, neuromodulatory synapses regulate relatively large neuronal populations and are believed to be important for learning and memory. Neuromodulators have been shown to influence LTP as well as LTD in animal and slice experiments in a non-linear manner (Kojic et al., 1997, Fujii et al., 2000, Matsuyama et al., 2000, Fujii and Sumikawa, 2001b, Mori et al., 2001, Huang et al., 2004b, Ge and Dani, 2005, Kemp and Manahan-Vaughan, 2005, Huang and Kandel, 2007, Luo et al., 2008, Costa et al., 2012, Park et al., 2012).

Human and animal studies have demonstrated an impact of the above-mentioned neuromodulatory substances on cognitive processes, motor functions, motor learning, attention, working and episodic memories (Provost and Woodward, 1991, Knecht et al., 2004, Winters and Bussey, 2005, Floel et al., 2008, Heishman et al., 2010, Mocking et al., 2012). Moreover, several neurological disorders show altered neuromodulator levels that usually lead to deficits in cognitive functions (Parkinson’s disease, schizophrenia, Alzheimer’s disease, Lewy body dementia, depression, etc), whose physiological basis might be impact of neuromodulators on plasticity.

In recent years several human studies were conducted using non-invasive brain stimulation techniques and pharmacological interventions to study the impact of neuromodulatory systems on different types of plasticity (Kuo et al., 2007, Kuo et al., 2008, Monte-Silva et al., 2009, Nitsche et al., 2009, Monte-Silva et al., 2010b, Thirugnanasambandam et al., 2012). In the studies presented in this thesis, we used different doses of pharmacologic agents to induce

alterations of cholinergic and serotonergic activity and different brain stimulation protocols to induce focal and non-focal plasticity in healthy human subjects.

1.4.1. Serotonin

The serotonergic system is one of the most important neuromodulatory systems in animals and humans, involved in many vital processes such as learning, memory, circadian rhythms and pain perception (Geyer, 1996, Hasbroucq et al., 1997, Jacobs and Fornal, 1997, Morin, 1999, Bert et al., 2008). Serotonin (5-HT) modulates neurotransmission by means of 5-HT receptors, which are a group of ligand-gated ion channels (5-HT3) and G-protein coupled receptors (5-HT1, 5HT-2, 5HT-4, 5HT-5, 5HT-6, and 5HT-7) (Nichols and Nichols, 2008). 5-HT receptor activation has been shown to modulate glutamate-and GABA-mediated neurotransmission (Ciranna, 2006), as well as to affect LTP and LTD induction (Kojic et al., 1997, Mori et al., 2001, Ryan et al., 2008). Serotonin also affects cholinergic, dopaminergic, and GABAergic neuromodulatory systems, that impact on plasticity and cognition (Consolo et al., 1994, Roerig and Katz, 1997, Gobert and Millan, 1999, Zaniewska et al., 2009).

Selective serotonin reuptake inhibitors (SSRIs) are one of the major classes of antidepressant drugs that inhibit the reuptake of serotonin by the presynaptic cell, therefore increasing its effect on the postsynaptic neuron (Stahl, 1998). Depression is thought to be affected by altered brain plasticity (Garcia, 2002, Christoffel et al., 2011) on which distress has a major impact (Caspi et al., 2003). Studies with animal models have shown that stress inhibits LTP and facilitates LTD induction (Foy et al., 1987, Xu et al., 1997, Rocher et al., 2004) and could be prevented by chronic SSRI administration (Holderbach et al., 2007). Serotonin enhancers have a positive effect on motor and cognitive functions in patients as well as healthy individuals (Dam et al., 1996, Loubinoux et al., 1999, Loubinoux et al., 2002a, Loubinoux et al., 2002b, Loubinoux et al., 2005, Chollet et al., 2011). This positive impact might be caused by serotoninergic modulation of cortical plasticity.

Recent studies in human subjects have shown that serotonin has a facilitatory impact on neuroplasticity. Single-dose SSRI administration enhanced anodal tDCS-induced facilitatory

plasticity and converted cathodal tDCS-induced inhibitory plasticity into facilitation (Nitsche et al., 2009). SSRI intake also enhanced facilitatory plasticity of early visual-evoked potentials and trendwise shifted inhibitory plasticity towards facilitation (Normann et al., 2007).

Other neuromodulators, such as dopamine, acetylcholine, and nicotine are characterized by a so-called "focusing effect" on focal, synapse-specific facilitatory plasticity (Kuo et al., 2007, Kuo et al., 2008, Monte-Silva et al., 2010b, Thirugnanasambandam et al., 2012), which explains their positive effect on processes that require consolidation of learning and memory-related cognitive functions, via increase of the signal-to-noise ratio. Unlike the above-mentioned neuromodulatory systems, data about the serotoninergic impact on focal neuroplasticity are missing. In accordance to the previous studies, we hypothesize that serotoninergic system activation should enhance focal facilitatory plasticity and abolish focal inhibitory plasticity or convert it into facilitation.

1.4.2. Nicotine

The cholinergic system is involved in attention, short-term memory, arousal and sensory perception (Provost and Woodward, 1991, Hahn and Stolerman, 2002, Kumari et al., 2003, Jubelt et al., 2008, Heishman et al., 2010). Pathological states of the cholinergic system are observed in schizophrenia and Alzheimer's disease (Jones et al., 1992, White and Levin, 1999). Cholinergic modulation is accomplished by means of two receptor types: nicotinic (nAChRs) and muscarinic acetylcholine receptors (mAChRs). NAChRs are ligand-gated cation channels that are non-selectively activated by acetylcholine and nicotine (Burnashev, 1998, Dajas-Bailador and Wonnacott, 2004). Besides addictive properties, several studies demonstrate positive effects of nicotine on cognitive functions (Hahn et al., 2002, Hahn and Stolerman, 2002, Jubelt et al., 2008, Froeliger et al., 2009, Mocking et al., 2012). Nicotine withdrawal often causes impairments of neuroplasticity and working/verbal memory in smoking individuals, while nicotine re-administration restores these functions (Jacobsen et al., 2005, Cole et al., 2010, Grundey et al., 2012a).

The physiological mechanism for the nicotinic modulation of cognition is thought to be its impact on neuroplasticity, accomplished by activation of nAChRs. $\alpha_4\beta_2$ and α_7 nAChRs modulate the permeability of Ca^{2+} ions, involved in LTD/LTP induction (Burnashev, 1998, Lisman, 2001). In accordance, several animal and slice studies have shown that nicotinic receptor activation results in LTP facilitation (Matsuyama et al., 2000, Fujii and Sumikawa, 2001a, Welsby et al., 2006, Nakauchi et al., 2007), reversal of GABAergic inhibition of LTP (Fujii et al., 2000) and LTD enhancement (Fujii and Sumikawa, 2001b, Ge and Dani, 2005).

Recent studies explored the nicotinic impact on cortical excitability and plasticity in humans. Global cholinergic activation preserved and prolonged both focal and non-focal inhibitory plasticity and increased focal, but abolished non-focal inhibitory plasticity. Nicotinic receptor activation induced similar effect for LTP-like plasticity, but LTD-like plasticity was abolished in healthy non-smoking subjects (Kuo et al., 2007, Thirugnanasambandam et al., 2012). These studies show a “focusing effect” of cholinergic activation on LTP-like plasticity, which can explain the positive cholinergic impact on cognition.

Nicotinic receptor activation was accomplished by application of nicotine patches in the above-mentioned study (Thirugnanasambandam et al., 2012), which non-specifically activates all nicotinic receptors, therefore contribution of specific nicotinic receptor subtypes to nicotinic modulation of neuroplasticity still remains unclear. Given that after-effects induced by tDCS and PAS are calcium-dependent (Stefan et al., 2002, Nitsche et al., 2003a), it can be hypothesized that nicotinic receptors with calcium channel properties ($\alpha_4\beta_2$ and α_7) are involved. Similar to the effect of nicotine, activation of these receptors should result in abolishment of non-focal plasticity and preservation of focal facilitatory plasticity in non-smoking humans.

1.5. Aim of the thesis

In the studies presented in this thesis, we aimed to explore the impact of modified brain stimulation protocols and neuromodulatory systems on stimulation-induced plasticity in humans. In the first study, we explored the intracortical and corticospinal effects of clinically

used 2mA direct current stimulation. We used several single and paired-pulse TMS protocols (single-pulse MEPs, motor thresholds, I-O curve, I waves, short-latency intracortical inhibition and intracortical facilitation, cortical silent period) to study its impact on various neurophysiological parameters. Based on the generally accepted assumption that stronger stimulation results in larger effects of tDCS (Nitsche and Paulus, 2000), we expected a positive correlation between intensity of stimulation and strength and duration of after-effects.

In the second study, we aimed to deepen our knowledge about serotoninergic modulation of neuroplasticity, specifically, its impact on PAS-induced focal plasticity. As PAS-induced plasticity is thought to share similarities with spike timing-dependent plasticity, results of this study could help us to explain the mechanisms of the positive serotoninergic impact on cognition and learning as well as on clinical symptoms in medical conditions, characterized by compromised and maladaptive plasticity (stroke, depression). For this purpose, we applied an experimental design similar to a previous study (Nitsche et al., 2009), with the only exception of the specific brain stimulation protocol (we administered PAS instead of tDCS). We expected that serotonin would shift plasticity towards an excitability enhancement.

In the last study, our goal was to explore the contribution of nicotinic acetylcholine receptor subtypes to neuroplasticity. Previous studies demonstrated an impact of global cholinergic and nicotinic receptor activation on stimulation-induced plasticity (Kuo et al., 2007, Thirugnanasambandam et al., 2012), but knowledge about the involvement of specific receptors in this process is limited. Therefore we aimed to focus on dose-dependent effect of $\alpha_4\beta_2$ nAChRs on both non-focal and focal types of plasticity. To that end, we administered different doses of the $\alpha_4\beta_2$ nicotinic receptor partial agonist varenicline on both, non-focal and focal plasticity-inducing brain stimulation protocols (tDCS and PAS). We expected that high drug dosages should demonstrate a focusing effect, similar to nicotine (Thirugnanasambandam et al., 2012), by preserving PAS-induced focal excitatory plasticity in non-smoking healthy individuals.

Chapter 2 – Original articles and manuscripts

2.1. Partially non-linear stimulation intensity-dependent effects of direct current stimulation on motor cortex excitability in humans

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Partially non-linear stimulation intensity-dependent effects of direct current stimulation on motor cortex excitability in humans

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Key points

- Application of 2 mA cathodal transcranial direct current stimulation for 20 min results in cortical excitability enhancement instead of inhibition.
- Longer or more intensive stimulation does not necessarily increase its efficacy.
- Short intracortical inhibition and facilitation are shifted towards excitability enhancement after both 2 mA anodal and cathodal stimulation.
- I-waves, input–output curves and cortical silent period are unaffected immediately after 2 mA stimulation.

Abstract Transcranial direct current stimulation (tDCS) of the human motor cortex at an intensity of 1 mA with an electrode size of 35 cm^2 has been shown to induce shifts of cortical excitability during and after stimulation. These shifts are polarity-specific with cathodal tDCS resulting in a decrease and anodal stimulation in an increase of cortical excitability. In clinical and cognitive studies, stronger stimulation intensities are used frequently, but their physiological effects on cortical excitability have not yet been explored. Therefore, here we aimed to explore the effects of 2 mA tDCS on cortical excitability. We applied 2 mA anodal or cathodal tDCS for 20 min on the left primary motor cortex of 14 healthy subjects. Cathodal tDCS at 1 mA and sham tDCS for 20 min was administered as control session in nine and eight healthy subjects, respectively. Motor cortical excitability was monitored by transcranial magnetic stimulation (TMS)-elicited motor-evoked potentials (MEPs) from the right first dorsal interosseous muscle. Global corticospinal excitability was explored via single TMS pulse-elicited MEP amplitudes, and motor thresholds. Intracortical effects of stimulation were obtained by cortical silent period (CSP), short latency intracortical inhibition (SICI) and facilitation (ICF), and I wave facilitation. The above-mentioned protocols were recorded both before and immediately after tDCS in randomized order. Additionally, single-pulse MEPs, motor thresholds, SICI and ICF were recorded every 30 min up to 2 h after stimulation end, evening of the same day, next morning, next noon and next evening. Anodal as well as cathodal tDCS at 2 mA resulted in a significant increase of MEP amplitudes, whereas 1 mA cathodal tDCS decreased corticospinal excitability. A significant shift of SICI and ICF towards excitability enhancement after both 2 mA cathodal and anodal tDCS was observed. At 1 mA, cathodal tDCS reduced single-pulse TMS-elicited MEP amplitudes and shifted SICI and ICF towards inhibition. No significant changes were observed in the other protocols. Sham tDCS did not induce significant MEP alterations. These results suggest that an enhancement of tDCS intensity does not necessarily increase efficacy of stimulation, but might also shift the

M.-F. Kuo and M. A. Nitsche contributed equally to this work.

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direction of excitability alterations. This should be taken into account for applications of the stimulation technique using different intensities and durations in order to achieve stronger or longer lasting after-effects.

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Abbreviations AMT, active motor threshold; CSP, cortical silent period; FDI, first dorsal interosseus; I–O, input–output; I-wave, indirect wave; ICF, intracortical facilitation; LTD, long-term depression; LTP, long-term potentiation; MEP, motor-evoked potential; MT, motor threshold; RMT, resting motor threshold; SICI, short latency intracortical inhibition; tACS, transcranial alternating current stimulation; TBS, theta burst stimulation; tDCS, transcranial direct current stimulation; TMS, transcranial magnetic stimulation; tRNS, transcranial random noise stimulation.

Introduction

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation technique that is able to induce polarity-dependent shifts of cortical excitability, which can last for approximately up to a few hours after stimulation with conventional protocols. Anodal tDCS depolarizes cortical neurons and increases their excitability, whereas cathodal tDCS is presumed to hyperpolarize neuronal membranes and decrease neuronal excitability. Pharmacological studies have shown that the long-lasting after-effects involve *N*-methyl-D-aspartate (NMDA) receptors and the GABAergic system (Liebetanz *et al.* 2002; Nitsche *et al.* 2003*a*, 2004*b*). The duration and strength of tDCS after-effects depend on duration and intensity of the applied current. The interdependency between these factors has been shown to be linear for a current strength of up to 1 mA (electrode size 35 cm^2) and a stimulation duration of up to 13 min (Nitsche & Paulus, 2000, 2001; Nitsche *et al.* 2003*b*).

In recent years tDCS has been increasingly used in functional studies in healthy humans, as well as clinical applications in patients suffering from neuropsychiatric diseases (Nitsche *et al.* 2008; Nitsche & Paulus, 2011). In these studies, stimulation duration and intensity has often been increased above the routine stimulation parameters based on an implicit assumption that longer stimulation duration or higher intensities will enhance efficacy of stimulation. Although these more intensive protocols have been shown to be effective in numerous studies (Fregni *et al.* 2006*a*; Ferrucci *et al.* 2009; Brunoni *et al.* 2011; Bueno *et al.* 2011), knowledge about their physiological effects is limited.

As non-linear effects of stimulation parameters on alterations of cortical excitability were demonstrated recently for other non-invasive brain stimulation protocols, such as theta burst stimulation (TBS), transcranial alternating current stimulation (tACS) and transcranial random noise stimulation (tRNS) (Doeltgen & Ridding, 2010; Gamboa *et al.* 2010; Moliadze *et al.* 2012), here we aimed to explore if increased intensity and prolongation of tDCS results in enhanced efficacy of stimulation with regard to polarity-dependent excitability alterations. We therefore administered 2 mA cathodal and anodal tDCS for 20 min to the primary motor cortex of healthy subjects, which is a frequently used stimulation protocol in cognitive and clinical studies (Iyer *et al.* 2005; Fregni *et al.* 2006*b*; Ferrucci *et al.* 2009; Brunoni *et al.* 2011; Ladeira *et al.* 2011). We explored the impact of these stimulation protocols on various parameters of corticospinal and intracortical excitability. The global change of corticospinal excitability in the motor cortex was measured by motor evoked potentials (MEPs) elicited by single-pulse transcranial magnetic stimulation (TMS), active and resting motor thresholds (MTs) and input–output (I–O) curves (Chen, 2000; Abbruzzese & Trompetto, 2002). Short latency intracortical inhibition (SICI) and facilitation (ICF) of motor cortex were explored by a paired-pulse TMS stimulation protocol, where a subthreshold conditioning stimulus is followed by a suprathreshold test pulse. The resulting increase or decrease of the MEP amplitude elicited by the test stimulus is determined by the respective interstimulus interval (ISI) (Kujirai *et al.* 1993). To monitor indirect waves (I-waves) generated by motor cortex stimulation as a parameter of the interaction between corticocortical circuits, another paired-pulse TMS protocol was used. Here a suprathreshold TMS test pulse was followed by a subthreshold one (Ziemann *et al.* 1998; Ziemann & Rothwell, 2000). The resulting change of MEP amplitude is specific for certain ISIs, reflecting cortical interactions between the interneuronal circuits. To study changes of cortical inhibition, furthermore the cortical silent period (CSP) was obtained (Fuhr *et al.* 1991; Bertasi *et al.* 2000; Romeo *et al.* 2000). Thus, ICF is determined by the glutamatergic system, whereas CSP and I-wave facilitation depend primarily on GABA (Paulus *et al.* 2008).

For 1 mA stimulation (stimulation duration 13 min anodal, 9 min cathodal tDCS), anodal DC stimulation enhanced single-pulse MEP amplitudes, slope of the I–O curve, intracortical facilitation and I-wave facilitation,

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Table 1. Subject characteristics

	Subjects						
Experimental session	*n*	Sex (M/F)	Age	RMT (%)*	AMT (%)*	SI_{1mv} (%)*	Baseline MEP amplitude (mV)
Experiment 1							
2 mA anodal	14	9 F/5 M	25.8 ± 3.7	40.1 ± 8.1	31.4 ± 7.2	49.2 ± 9.8	0.96 ± 0.13
2 mA cathodal	14	9 F/5 M	25.8 ± 3.7	40.1 ± 7.4	32.7 ± 7.6	49.4 ± 8.7	0.99 ± 0.07
Experiment 2							
1 mA cathodal	9	6 F/3 M	26 ± 4.5	43.6 ± 8.5	33.3 ± 7.8	53.1 ± 9.5	1.005 ± 0.15
Experiment 3							
Sham	8	6 F/2 M	26.9 ± 2.6	—	32.1 ± 9.4	51.6 ± 12.7	0.93 ± 0.03

Data are presented as mean ± SD; *n* = number of participants; F = female; M = male; RMT = resting motor threshold; AMT = active motor threshold; SI_{1mv} = TMS intensity adjusted to elicit ~1 mV peak-to-peak amplitude of motor evoked potentials (MEPs).
*Percentage of maximum stimulator output.

while cathodal tDCS had grossly antagonistic effects in previous studies (Nitsche *et al.* 2005). Because 2 mA cathodal tDCS applied for 20 min resulted in excitability-enhancing effects, we added two control experiments with 1 mA and sham stimulation for the same duration to explore the dependency of this effect from stimulation intensity, and rule out any unspecific effects depending on the time course of the study or tDCS-related arousal.

Methods

Subjects

Twenty-one healthy subjects aged 26.28 ± 3.4 years (7 males/14 females) (for details see Table 1) were recruited. All subjects were right-handed according to the Edinburgh handedness inventory (Oldfield, 1971). None of them took any medication, or had a history of neurological diseases, pregnancy or metallic head implants. They all gave written informed consent and were compensated for participation. Subjects were blinded for stimulation conditions. The investigation was approved by the Ethics Committee of the University of Göttingen, and conforms to the principles laid down in the Declaration of Helsinki.

tDCS

Direct current was applied through a pair of saline-soaked surface sponge electrodes (100 and 35 cm^2) and delivered by a battery-driven constant current stimulator (neuro-Conn GmbH, Ilmenau, Germany). The motor cortex electrode (35 cm^2) was fixed over the area representing the right first dorsal interosseus (FDI) muscle as identified by TMS, and the other electrode (100 cm^2) was placed contralaterally above the right orbit. tDCS was applied for 20 min, with current ramped up and down to and from 2 mA or 1 mA over 8 s. The intensities correspond to current densities of 0.057 mA cm^{-2} (2 mA/35 cm^2) and 0.029 mA cm^{-2} (1 mA/35 cm^2) under the active electrodes and 0.02 mA cm^{-2} (2 mA/100 cm^2) and 0.01 mA cm^{-2} (1 mA/100 cm^2) under the reference electrodes for 2 and 1 mA conditions, respectively. During sham stimulation, the current was ramped up for 20 s, followed by 30 s of 2 mA stimulation, and then it was ramped down for 10 s. The polarity for sham stimulation was randomized (Ambrus *et al.* 2012). Twenty minutes after the beginning of sham tDCS, the stimulation electrodes were removed and TMS measurements were taken. The minimum period between sessions for a single subject was 7 days, and sessions were applied in randomized order.

Monitoring of motor cortical excitability

MEPs were induced in the right FDI by single-pulse TMS over the left primary motor cortex, conducted by a Magstim 200 magnetic stimulator (Magstim, Whiteland, Dyfed, UK) with a figure-of-eight magnetic coil (diameter of one winding, 70 mm; peak magnetic field, 2.2 T). For the paired-pulse TMS protocols, the coil was connected to two Magstim 200 stimulators via a bistim module. The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline. The optimal coil placement (hotspot) was defined as the site where TMS resulted consistently in the largest MEPs of the contralateral FDI. Surface MEPs were recorded from the right FDI with Ag-AgCl electrodes in a belly-tendon montage. The signals were amplified, and band-pass filtered (2 Hz to 2 kHz; sampling rate, 5 kHz). Signals were digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK), controlled by Signal Software (Cambridge Electronic Design, v. 2.13) and stored for offline analysis. A waterproof pen was used to mark the positions of TMS coil and FDI electrodes to ensure that they were positioned at the same spot during the whole experimental session.

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Motor threshold determination

Resting motor threshold (RMT) was determined as the minimum stimulator output needed to elicit an MEP response of 50–100 μV in the relaxed FDI muscle in at least three of six consecutive trials. The active motor threshold (AMT) was the minimum intensity needed to elicit an MEP response of ~200–300 μV during moderate spontaneous background muscle activity (~15% of the maximum muscle strength) in at least three of six consecutive trials.

Single-pulse MEPs (1 mV)

Single-pulse MEPs were recorded with the TMS intensity adjusted to elicit ~1 mV peak-to-peak amplitude (SI_{1mV}) at baseline. Stimulation intensity was kept constant for the post-stimulation assessment.

Input–output curve

The I–O curve was determined using TMS intensities of 100, 110, 130 and 150% RMT (15 stimuli per block).

Intracortical inhibition and facilitation

Intracortical inhibition and facilitation were obtained by a TMS paired-pulse protocol including ISIs of 2, 3, 5, 10 and 15 ms (Kujirai *et al.* 1993). The first three ISIs represent inhibitory and the last two ISIs facilitatory intervals. The exact interval between the paired pulses was randomized (4 ± 0.4 s). In this protocol a subthreshold conditioning stimulus was applied (determined as 70% of AMT), followed by a second suprathreshold test stimulus. The test stimulus was adjusted to achieve a baseline MEP of ~1 mV and readjusted during the respective stimulation protocols, if needed, to compensate for the effects of tDCS-caused corticospinal excitability changes on test pulse amplitude. The pairs of stimuli were organized in blocks in which each ISI and one test pulse was represented once and were pseudorandomized. These blocks were repeated 15 times. Blocks of MEPs in which the muscle was not relaxed were excluded from the analysis.

I-wave facilitation

I-wave facilitation was measured using a TMS paired-pulse protocol including ISIs of 1.1, 1.3, 1.5, 2.3, 2.5, 2.7, 2.9, 4.1, 4.3 and 4.5 ms (Ziemann *et al.* 1998). In this protocol the TMS test stimulus precedes the conditioning stimulus (determined as 70% of RMT). The test stimulus was adjusted to achieve a baseline MEP of ~1 mV and readjusted during the respective stimulation protocols, if needed, to compensate for the effects of corticospinal excitability changes on test pulse amplitude. The pairs of stimuli were organized in blocks in which each ISI and one test pulse was represented once and were pseudo-randomized. These blocks were repeated 15 times. Blocks of MEPs in which the muscle was not relaxed were excluded from the analysis.

Cortical silent period

CSP was measured in the voluntarily contracted (~15% of the maximum muscle strength) FDI muscle. For eliciting CSP, TMS was applied at an intensity of SI_{1mV} and 120% RMT, each for 10 consecutive recordings. Latency and duration of CSP were calculated from the time of the stimulus onset to the reappearance of voluntary muscle activity (Fuhr *et al.* 1991; Bertasi *et al.* 2000; Romeo *et al.* 2000).

Experimental procedures

Experiment 1. The volunteers were seated in a comfortable chair with head and arm rests. First, the hotspot (the coil position that produced the largest MEPs of the right FDI) was identified by TMS. Then the stimulation intensity was adjusted to elicit single-pulse MEPs with peak-to-peak amplitudes of an average of 1 mV and 20 MEPs were recorded. After determination of SI_{1mv}, RMT and AMT were obtained. After measuring AMT, a 15 min break followed to avoid an effect of muscle contraction on the next measurements. After this break the following parameters were measured: I–O curves, I-waves, intracortical inhibition and facilitation, and CSP. The order of measurement of these parameters was randomized, except of that of CSP, which was obtained always at the end of this block, as it required a consecutive ~20 min break because of long-lasting voluntary muscle contraction. After this break, 2 mA cathodal or anodal tDCS was administered for 20 min and immediately after removal of the tDCS electrodes single-pulse MEPs were recorded, and resting and active MTs were obtained. The other parameters (I–O curves, I-waves, SICI-ICF, CSP) were then measured. For the latter protocols, TMS intensity was readjusted to obtain single test pulse amplitudes of 1 mV, if needed. Further TMS measurements (MEPs at SI_{1mV}, motor thresholds and SICI-ICF only) were conducted every 30 min up to 2 h after the end of tDCS, in the evening of the same day (SE), the next morning at ~09:00 h (NE), next noon at ~12:00 h (NN) and next evening at ~18:00 h (NE) (Fig. 1).

Experiment 2. Due to results of Experiment 1, we decided to conduct a control experiment using the identical study design with 1 mA cathodal tDCS. Nine of 14 subjects from

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Experiment 1 participated in Experiment 2. In this session, no TMS measurements were performed on the second day. Results of this experiment were compared with the results from 2 mA cathodal tDCS of Experiment 1.

Experiment 3. A control experiment was conducted using sham tDCS. Eight subjects were recruited for this session. Single-pulse MEPs, AMTs and SICI-ICF were measured before tDCS, immediately after, and 30 and 60 min after the end of tDCS.

Analysis and statistics

Experiment 1. To compare MTs, the inter-individual means of the TMS intensity at AMT and RMT were calculated for the before and after-stimulation conditions separately. A repeated measures analysis of variance (ANOVA) was performed on the above-mentioned data using AMT/RMT value as the dependent variable, and polarity of stimulation and time course as independent within-subject factors. For significant ANOVA results, for all conditions values before tDCS were compared with those after tDCS using *post hoc* Student's *t* tests (paired samples, two-tailed, $P < 0.05$).

For the single-pulse TMS conditions, the individual means of 20 MEP amplitudes were calculated for all subjects and the after-stimulation mean MEP amplitudes were normalized to the respective mean baseline MEP amplitudes. Grand averages for each time point were then calculated. A repeated-measures ANOVA was performed on the above-mentioned data using MEP amplitude as the dependent variable, and polarity of stimulation and time course as within-subject factors. For I–O curves, TMS intensity served as an additional within-subject factor. For significant ANOVA results, *post hoc* comparisons were performed using Student's *t* tests (paired samples, two-tailed, $P < 0.05$).

For the paired-pulse stimulation protocols, the resulting mean values were normalized to the respective single-pulse condition. First intra-, and then inter-individual means were calculated for each condition. To determine significant changes, repeated measures ANOVAs were performed (ISIs, polarity of stimulation and time course as independent within-subject factors and MEP amplitude as dependent variable) (Table 2). In case of significant results of ANOVA, *post hoc* comparisons were performed using Student's t tests (paired samples, two-tailed, $P < 0.05$) to compare mean MEP amplitudes at time points after plasticity induction *vs.* the respective baseline values for the respective ISIs.

For the CSP protocol, individual means of CSP durations were calculated for all subjects both at the intensity of SI_{1mV} and at 120% RMT and the after-stimulation CSP values were normalized to respective mean baseline CSP durations. A repeated-measures ANOVA was performed on the above-mentioned data using CSP duration as the dependent variable, and polarity of stimulation, TMS

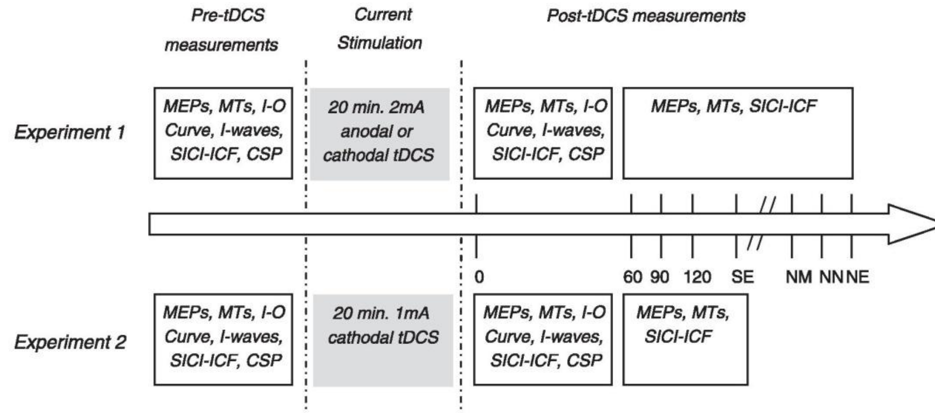

Figure 1. Course of the study
In the beginning of each session, 20 baseline single-pulse MEPs of SI_{1mv} intensity, resting motor threshold (RMT), active motor threshold (AMT), input–output (I–O) curve, I-waves, short-latency intracortical inhibition, intracortical facilitation (SICI-ICF) and cortical silent period (CSP) were recorded. Afterwards, 2 or 1 mA tDCS over 20 min was administered and then the above-mentioned parameters were recorded again. From 60 min after the stimulation, single- and double-pulse TMS parameters were recorded as follows: single-pulse MEPs of SI_{1mv} intensity, RMT, AMT and SICI-ICF 60, 90 and 120 min after the end of tDCS and at the evening on the same day (∼18:00; SE = same evening). For Experiment 1 we also performed these measurements on the next morning (∼9:00; NM), next noon (∼12:00; NN) and next evening (∼18:00; NE).

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Table 2. Repeated-measures ANOVA results for single- and paired-pulse protocols

Measurement	Factor	d.f.	*F*	*P*
Experiment 1				
MEP	TDCS	1	0.155	0.702
	Time	8	5.394	<0.001*
	TDCS × Time	8	1.761	0.096
RMT	TDCS	1	1.792	0.204
	Time	8	0.782	0.620
	TDCS × Time	8	0.971	0.463
AMT	TDCS	1	0.001	0.975
	Time	8	1.335	0.234
	TDCS × Time	8	0.694	0.696
I–O curve	TDCS	1	1.239	0.286
	Time	1	0.340	0.570
	Intensity	3	52.650	<0.001*
	TDCS × Time	1	0.013	0.909
	TDCS × Intensity	3	1.442	0.245
	TIME × Intensity	3	0.237	0.870
	TDCS × Time × Intensity	3	2.385	0.084
SICI-ICF	TDCS	1	0.378	0.549
	time	8	1.929	0.063
	ISI	4	20.949	<0.001*
	TDCS × Time	8	2.102	0.042*
	TDCS × ISI	4	1.310	0.279
	Time × ISI	32	1.141	0.277
	TDCS × Time × ISI	32	1.005	0.463
I-wave facilitation	TDCS	1	1.911	0.190
	Time	1	0.334	0.573
	ISI	9	17.574	<0.001*
	TDCS × Time	1	0.207	0.657
	TDCS × ISI	9	0.343	0.959
	TIME × ISI	9	0.460	0.899
	TDCS × Time × ISI	9	0.894	0.533
CSP	TDCS	1	0.590	0.456
	Intensity	1	0.115	0.740
	Time	1	0.034	0.856
	TDCS × Intensity	1	0.696	0.419
	TDCS × Time	1	0.590	0.456
	Intensity × Time	1	0.115	0.740
	TDCS × Intensity × Time	1	0.696	0.419
Experiment 2				
MEP	TDCS	1	19.018	**0.003***
	Time	5	1.329	0.275
	TDCS × Time	5	2.657	**0.039***
RMT	TDCS	1	4.659	0.063
	Time	5	1.804	0.134
	TDCS × Time	5	0.904	0.488
AMT	TDCS	1	0.620	0.454
	Time	5	1.894	0.117
	TDCS × Time	5	0.924	0.476

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Table 2. Continued

Measurement	Factor	d.f.	F	P
I–O curve	TDCS	1	1.356	0.257
	Time	1	1.239	0.298
	Intensity	3	38.440	**<0.001***
	TDCS × Time	1	0.790	0.400
	TDCS × Intensity	3	0.549	0.654
	Time × Intensity	3	1.126	0.358
	TDCS × Time × Intensity	3	0.575	0.637
SICI-ICF	TDCS	1	1.051	0.339
	Time	5	4.106	**0.005***
	ISI	4	9.853	**<0.001***
	TDCS × Time	5	0.981	0.443
	TDCS × ISI	4	0.502	0.735
	Time × ISI	20	0.787	0.726
	TDCS × Time × ISI	20	1.273	0.207
I-wave facilitation	TDCS	1	0.895	0.372
	Time	1	2.200	0.176
	ISI	9	20.922	**<0.001***
	TDCS × Time	1	0.014	0.909
	TDCS × ISI	9	1.115	0.364
	Time × ISI	9	1.347	0.229
	TDCS × Time × ISI	9	0.691	0.715
CSP	TDCS	1	3.679	0.091
	Intensity	1	1.561	0.247
	Time	1	0.360	0.565
	TDCS × Intensity	1	3.596	0.094
	TDCS × Time	1	3.679	0.091
	Intensity × Time	1	1.561	0.247
	TDCS × Intensity × Time	1	3.596	0.094
Experiment 3				
MEP	Time	3	0.142	0.934
AMT	Time	3	0.237	0.870
SICI-ICF	Time	3	0.123	0.945
	ISI	4	3.225	**0.027***
	Time × ISI	12	1.358	0.203

MEP = motor-evoked potential; RMT = resting motor threshold; AMT = active motor threshold; SICI-ICF = short-latency intracortical inhibition and intracortical facilitation; CSP = cortical silent period.
*$P < 0.05$.

intensity and time course as independent within-subject factors.

To exclude differences between baseline values of different tDCS conditions, for both single- and double-pulse protocols, we compared the respective values using Student's t tests. The Mauchly test of sphericity was performed and the Greenhouse–Geisser correction was applied when necessary.

Experiment 2. For Experiment 2, calculations were identical to those of Experiment 1, the only exception being that stimulation intensity was used as independent within-subject factor instead of polarity of stimulation.

Experiment 3. For Experiment 3, calculations were identical to those of Experiment 1, the only exception being that stimulation polarity was not used as an independent within-subject factor.

Results

Subjects reported similar itchy sensations at the skin during both 2 mA cathodal and anodal trials, but these sensations were weaker during 1 mA cathodal tDCS. Baseline values of MEPs, MTs and CSPs did not differ significantly between stimulation conditions.

Experiment 1

Motor thresholds. Baseline RMT was 40.1 ± 8.1% (all values are reported as means ± standard error of the mean

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(SEM)) of maximum stimulator output for 2 mA cathodal and 40.1 ± 7.4% for 2 mA anodal stimulation; AMT was 31.4 ± 7.2 and 32.8 ± 7.6%, respectively. Baseline values did not differ between stimulation conditions. For the after-tDCS conditions, the ANOVA results were not significant (results of respective ANOVAs of Experiment 1 and 2 are shown in Table 2).

Single-pulse MEPs (1 mV). Baseline MEP values were 0.96 ± 0.13 mV for 2 mA anodal and 0.99 ± 0.07 for 2 mA cathodal stimulation obtained by 49.4 ± 8.7 and 49.2 ± 9.8% of maximum stimulator output, respectively. Baseline values did not differ between stimulation conditions. ANOVA revealed a significant main effect of time after stimulation ($F_8 = 5.378$, $P < 0.001$). The results of the *post hoc* tests showed a significant increase of MEP amplitudes at 60 and 90 min after 2 mA anodal and 90 and 120 min after 2 mA cathodal stimulation ($P < 0.05$) (Fig. 2, for the results obtained for non-standardized MEP amplitudes, see supplementary Fig. S1 and Table S1).

Input–output curve. The slope of the I–O curve was not changed by either cathodal or anodal 2 mA stimulation. ANOVA showed a significant effect of TMS Intensity ($F_3 = 52.650$, $P < 0.001$), but no significant interaction between tDCS, Time and TMS Intensity. Baseline values did not differ between stimulation conditions.

Intracortical inhibition and facilitation

ANOVA showed significant effects of ISI ($F_4 = 20.929$, $P < 0.001$) and tDCS × Time ($F_8 = 2.102$, $P = 0.042$). *Post hoc* Student's *t* tests (paired, two-tailed, $P < 0.05$) show that both 2 mA cathodal and anodal stimulation shifted cortical excitability towards an enhancement of excitability. At 2 mA, anodal tDCS increased facilitation for an ISI of 10 ms immediately after stimulation and decreased inhibition for an ISI of 5 ms both immediately, and 60 and 90 min after stimulation. A similar increase of facilitation for an ISI of 10 ms and decrease of inhibition for an ISI of 5 ms was observed 90 and 120 min after 2 mA cathodal stimulation (Fig. 3*A* and *B*). Baseline values did not differ between stimulation conditions.

I-wave facilitation. ANOVA revealed a significant main effect of ISI ($F_9 = 17.574$, $P < 0.001$), but no significant interaction between tDCS, Time and ISI. Both 2 mA anodal and cathodal stimulations resulted in no change of the respective I-wave peaks. Baseline values did not differ between stimulation conditions.

Cortical silent period. Average baseline CSP durations were 0.136 ± 0.027 and 0.141 ± 0.032 s for 2 mA anodal, and 0.14 ± 0.025 and 0.147 ± 0.031 s for 2 mA cathodal

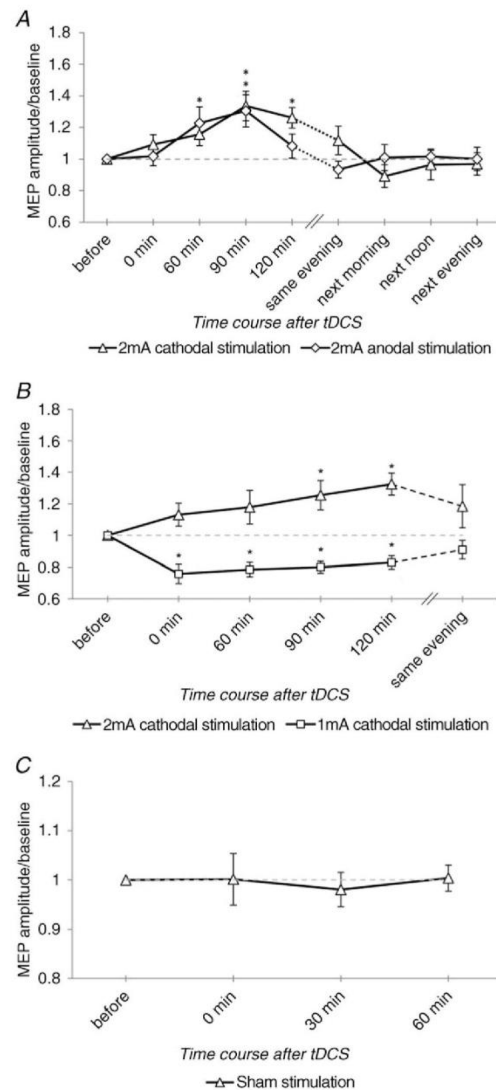

Figure 2. After-effects of anodal and cathodal tDCS on single-pulse MEP amplitudes
A–C, after-effects of (*A*) 2 mA anodal and 2 mA cathodal tDCS (number of participants = 14), (*B*) 2 mA cathodal and 1 mA cathodal tDCS (number of participants = 9) and (*C*) sham tDCS (number of participants = 8) on the single-pulse MEP amplitudes (means ± SEM) at the TMS intensity which elicited 1 mV MEP amplitudes at baseline. Asterisks indicate significant differences of MEP amplitudes from baseline values ($P < 0.05$). Anodal stimulation at 2 mA shows a significant increase of MEP amplitudes 60 and 90 min after stimulation, compared with 2 mA cathodal stimulation 90 and 120 min after tDCS. Cathodal stimulation at 1 mA shows a significant decrease in MEP amplitudes at 0–120 min after stimulation. Sham tDCS did not induce any significant changes.

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stimulation for 120% RMT and SI_{1mV} TMS intensities, respectively. ANOVA showed no significant change in CSP duration and also no interaction between TDCS, Intensity and Time. Baseline values did not differ between stimulation conditions.

Experiment 2

Motor thresholds. Baseline MTs in this experiment did not differ significantly from the respective values of Experiment 1. RMT was 43.6 ± 8.5% and AMT was 33.3 ± 7.8% of maximum stimulator output. ANOVA for the 2 and 1 mA cathodal stimulation conditions was not significant.

Single-pulse MEPs (1 mV). The average baseline MEP value was 1.005 ± 0.15 mV obtained by 53.1 ± 9.5% of maximum stimulator output. MEP amplitude and stimulation intensity did not differ significantly from that of Experiment 1. ANOVA for the 2 and 1 mA cathodal stimulation conditions revealed a significant main effect of tDCS ($F_1 = 23.691$, $P < 0.001$) and tDCS × TIME

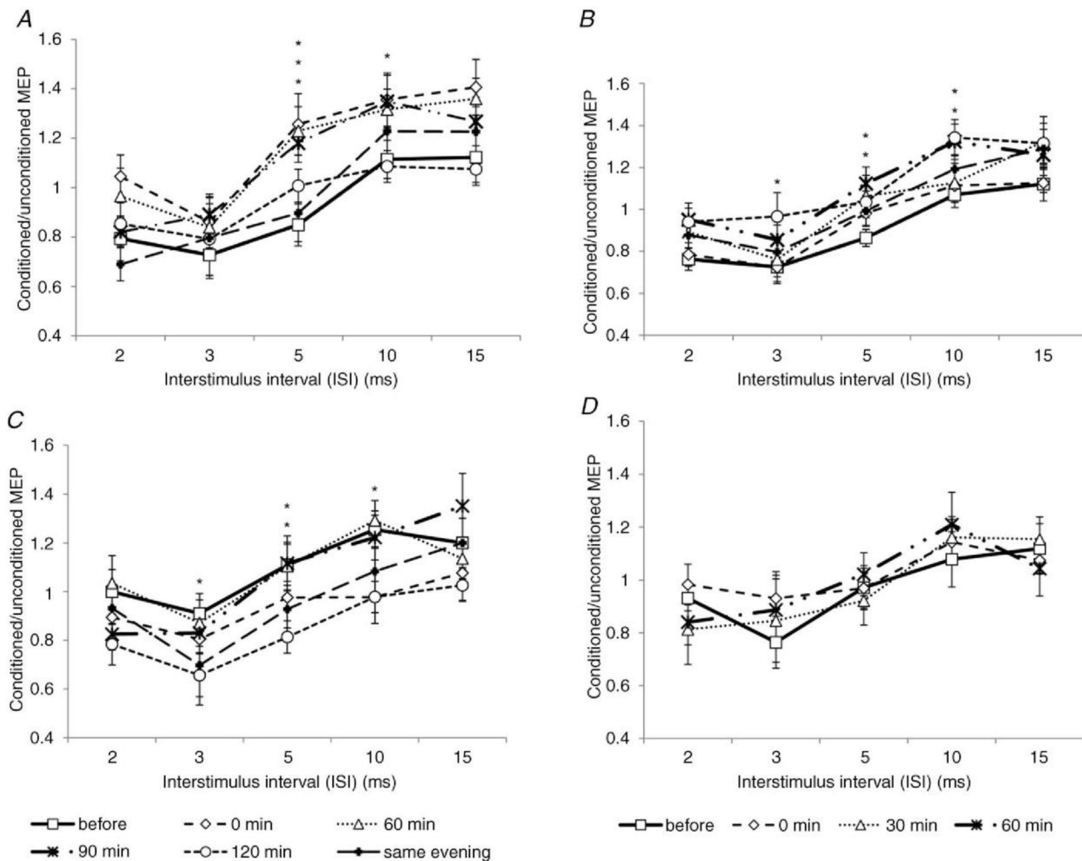

Figure 3. Intracortical inhibition and facilitation is modulated by tDCS
A–D, single-pulse standardized double stimulation MEP amplitude ratios ± SEM are depicted for ISIs revealing inhibitory (ISIs of 2, 3 and 5 ms) and facilitatory (ISIs of 10 and 15 ms) effects for (*A*) 2 mA anodal, (*B*) 2 mA cathodal, (*C*) 1 mA cathodal and (*D*) sham tDCS. Anodal tDCS at 2 mA decreases inhibition and increases facilitation immediately after stimulation for ISIs of 5 and 10 ms and 60 and 90 min after stimulation for an ISI of 5 ms; similar effects were observed 90 and 120 min after 2 mA cathodal tDCS. After 1 mA cathodal tDCS, facilitation is decreased for an ISI of 10 ms immediately after stimulation and inhibition is increased for ISIs of 5 ms at 90 min and 3 and 5 ms at 120 min after stimulation. Sham tDCS did not induce any significant changes. Asterisks indicate significant differences of standardized double stimulation MEP amplitudes from respective before stimulation values ($P < 0.05$).

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interaction ($F_5 = 4.141$, $P < 0.003$). The results of the *post hoc* Student's *t* tests showed a significant decrease of MEP amplitudes after 1 mA cathodal stimulation as compared to baseline and an excitability increase after 2 mA cathodal stimulation for 120 min after tDCS ($P < 0.05$) (Fig. 2).

Input–output curve. ANOVA for 2 and 1 mA cathodal tDCS showed a significant effect for TMS intensity ($F_3 = 38.440$, $P < 0.001$), but no significant interaction between tDCS, Time and TMS Intensity. A non-significant tendency towards a decrease of the I–O curve slope can be observed for the 1 mA condition (Fig. 4). MEP amplitudes were 1.91 ± 1.33 and 2.73 ± 1.83 mV before stimulation and 1.55 ± 0.77 and 2.32 ± 1.27 mV after stimulation at intensities of 130 and 150% of RMT, respectively.

Intracortical inhibition and facilitation. ANOVA showed a significant effect of ISI ($F_4 = 9.853$, $P < 0.001$) and Time ($F_5 = 4.106$, $P = 0.005$). For 1 mA cathodal tDCS, intracortical facilitation (ISI 10 ms) decreased immediately after stimulation and inhibition (ISIs of 3 and 5 ms) increased significantly 90 and 120 min after the end of stimulation, compared to the respective baseline values, as shown by the *post hoc* Student's *t* tests ($P < 0.05$) (Fig. 3*C*). Baseline values did not differ between stimulation conditions.

I-wave facilitation. ANOVA for the 2 and 1 mA cathodal stimulation showed a significant main effect for ISI ($F_9 = 18.068$, $P < 0.001$), but no significant interaction between tDCS, Time and ISI. Baseline values did not differ between stimulation conditions.

Cortical silent period. Average baseline CSP values were 0.138 ± 0.03 and 0.146 ± 0.027 s for 120% RMT and SI_{1mV} TMS intensities, respectively, and did not differ significantly from those of Experiment 1. ANOVA for the 2 and 1 mA cathodal tDCS showed no significant change in CSP duration and no interaction between tDCS, Intensity and Time.

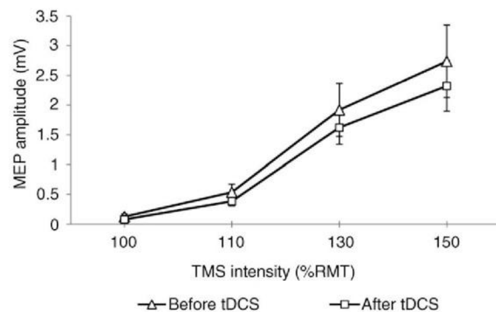

Figure 4. Effect of 1 mA cathodal tDCS on input–output curve
MEP amplitudes (means ± SEM) are displayed before and after application of 1 mA cathodal tDCS. A trend towards a decrease of MEP amplitudes after tDCS can be observed, in line with a previous study of our group (Nitsche *et al.* 2005).

Experiment 3

Active motor threshold. Baseline AMT values in this experiment did not differ significantly from the respective values of Experiment 1. AMT was 32.1 ± 9.4% of maximum stimulator output. The ANOVA results were not significant.

Single-pulse MEPs (1 mV). The average baseline MEP value was 0.93 ± 0.03 mV obtained by 51.6 ± 12.7% of maximum stimulator output. MEP amplitude and stimulation intensity did not differ significantly from that of Experiment 1. ANOVA did not reveal significant main effect of Time ($F_3 = 0.142$, $P = 0.93$) (Fig. 2*C*).

Intracortical inhibition and facilitation. ANOVA showed a significant effect of ISI ($F_4 = 3.225$, $P = 0.027$) but no significant interaction between Time and ISI (Fig. 3*D*).

Discussion

Cathodal stimulation, so far thought to be the cornerstone in producing cortical inhibition by tDCS, loses this property with double intensity and instead induces excitation. The results of the present study show that opposing directions of plasticity are no longer warranted at 2 mA tDCS for 20 min. As this stimulation has recently become increasingly used in clinical studies and some positive effects have been achieved, it is important to study its physiological effects. Based on previous experiments with 1 mA stimulation (Nitsche & Paulus, 2001; Nitsche *et al.* 2003*b*) we expected a direct correlation between stimulation intensity and time. In contrast, the increase of intensity and duration of stimulation did not uniformly produce a stronger effect. To rule out the possibility that this effect was due to the specific subject group explored, we performed 1 mA cathodal stimulation for 20 min in nine subjects of the same group. Here the results were similar to those described in previous studies, where application of 1 mA cathodal tDCS for 18 min resulted in a decrease of single-pulse MEP amplitudes lasting for up to 120 min after stimulation and a SICI–ICF shift towards reduced intracortical excitability (Nitsche *et al.* 2005; Monte-Silva *et al.* 2010). Furthermore, sham tDCS did not induce significant MEP alterations, which ruled out an unspecific effect of 2 mA tDCS on motor cortex excitability.

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Effects of tDCS on corticospinal excitability

Effect of tDCS on single-pulse MEPs. Anodal stimulation at 2 mA resulted in an excitability enhancement which lasted up to 90 min after stimulation, comparable to the after-effects of 13 min 1 mA anodal stimulation (Nitsche & Paulus, 2001). In contrast, 2 mA cathodal tDCS induced qualitatively different effects, as compared to previous studies with 1 mA cathodal tDCS (Nitsche *et al.* 2003*b*; Monte-Silva *et al.* 2010). Interestingly, other recently conducted studies applying different plasticity-inducing stimulation protocols also show a non-linear association between stimulation intensities and the direction of the resulting after-effects (Doeltgen & Ridding, 2010; Moliadze *et al.* 2012). For theta burst transcranial magnetic stimulation (TBS) it was demonstrated that a short duration continuous TBS applied with an intensity of 65% of RMT induced cortical inhibition, whereas the same technique at an intensity of 70% RMT resulted in an excitability enhancement (Doeltgen & Ridding, 2010). Another study demonstrated that tACS and tRNS reduced cortical excitability at an intensity of 0.4 mA and enhanced it at an intensity of 1 mA (Moliadze *et al.* 2012). One possible mechanism for these reversed effects might be the dependency of the direction of plasticity from the amount of neuronal calcium influx caused by the respective stimulation protocol, as shown primarily in animal models so far. Thereby, low postsynaptic calcium enhancement causes long-term depression (LTD), whereas large calcium increases result in long-term potentiation (LTP; Cho *et al.* 2001; Lisman, 2001). Thus, it might be speculated that the larger stimulation intensity in the case of 2 mA cathodal tDCS, and the stronger TBS, tACS and tRNS protocols increase calcium level to an amount that induces LTP-like plasticity, whereas lower stimulation intensity results in a lower, LTD-like plasticity-generating calcium level. Accordingly, the after-effects of tDCS are caused by calcium-dependent mechanisms (Nitsche *et al.* 2003*a*). It has also been demonstrated that doubling the stimulation duration from 13 to 26 min shifts the 1 mA anodal tDCS-induced after-effects to excitability diminution, and that this effect is calcium-dependent (Monte-Silva *et al.* in press). Further evidence for non-linear effects of tDCS, which might be calcium-dependent, originates from pharmacological studies, where a serotonine reuptake inhibitor and a D2/D3 receptor agonist at high dosage switched the 1 mA cathodal tDCS-induced after-effect to excitation (Nitsche *et al.* 2009). Another possible mechanism explaining excitatory after-effects of 2 mA cathodal tDCS could be that DC stimulation induces de- and hyperpolarization via hyperpolarizing the soma and depolarizing dendrites, respectively, with cathodal stimulation (Jefferys, 1981; Ghai *et al.* 2000; Bikson *et al.* 2004). Moreover, the resulting neuronal excitability change is determined by the axonal orientation relative to the electric field vector, from which it follows that tDCS-induced homogenous electric fields do not uniformly modulate all neurons in the stimulated area (Kabakov *et al.* 2012). Doubling current intensity in the case of 2 mA cathodal tDCS could therefore have increased dendritic depolarization to a level which has an impact on neuronal excitability or resulted in polarization of structures with different neuronal orientation, therefore producing plasticity different from that of 1 mA tDCS. Furthermore, due to modelling and imaging studies the current injected by tDCS with conventional electrode montages affects several regions of the brain (Datta *et al.* 2009), also beyond the target area, and changes functional connectivity between them (Polania *et al.* 2011*a*,*b*). An increase in the intensity of injected current should proportionally increase the electric field in every affected brain region, and might lead to recruitment of other non-target brain regions, which could indirectly affect and change the direction of plasticity in the target regions. In accordance, it has been demonstrated that 1 mA anodal tDCS over the premotor cortex decreases intracortical inhibition and increases facilitation in the primary motor cortex (Boros *et al.* 2008). Moreover, it was shown that the inhibitory ventral premotor to primary motor cortex pathway can be changed to excitatory in a state-dependent manner after paired associative stimulation of premotor and motor cortices (Davare *et al.* 2009; Buch *et al.* 2011). At present, however, all of these explanations are speculative, and should be explored in future studies directly.

Interestingly, in contrast to the conventional 1 mA stimulation protocols, 2 mA stimulation induces after-effects with a delay. There is no clear explanation for this delayed effect so far, although it has been observed in animal studies, and also for other non-invasive plasticity induction protocols in humans (Bindman *et al.* 1964; Bi & Poo, 1998; Stefan *et al.* 2000) and under lorazepam-reinforcing GABAergic contribution for tDCS (Nitsche *et al.* 2004*b*). Possible reasons for this delay might be transient homeostatic counter-regulation, alterations of intracellular calcium, and different neuronal populations affected by 2 mA, as compared to the 1 mA stimulation protocols, because stronger protocols should affect deeper cortical layers, and might also generate plasticity in other types of neurons (Purpura & McMurtry, 1965).

No effect on MTs. Both 2 mA cathodal and anodal, as well as 1 mA cathodal tDCS did not change motor thresholds, just as after application of 1 mA tDCS in a former study (Nitsche *et al.* 2005), which was explained by major tDCS effects on cortical neurons, while MTs depend primarily on corticospinal neurons. Moreover, the spatial disparity between the large tDCS electrode, which should affect many more neurons than those are affected by motor threshold determination, might have prevented significant effects. Only one study reported an RMT increase after

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1.5 mA cathodal tDCS for 10 min (Ardolino *et al.* 2005), but still inducing inhibitory after-effects at this amplitude.

No effect on I–O curve. Both 2 mA cathodal and anodal stimulation resulted in no change of the I–O curve slope, which was obtained only once immediately after the end of tDCS. These results are not in accordance with those of a previous study with 1 mA protocols (Nitsche *et al.* 2005). This discrepancy is most probably caused by the fact that tDCS in the current study induced delayed after-effects evolving not immediately after stimulation, as can be seen also by the other parameters obtained in the present study. In the 1 mA cathodal stimulation condition, which induced after-effects without a prominent delay, we saw a tendency towards the decrease of I–O curve slope, which is similar to the results of the above-mentioned study. The non-significant trend after 1 mA cathodal tDCS is most probably a result of the higher variability in the present as compared to the previous study caused by the lower number of subjects and randomized order of measurements before and after stimulation.

Effects of tDCS on intracortical excitability

SICI and ICF are affected by tDCS. For 2 mA anodal tDCS, short latency intracortical inhibition and facilitation are shifted towards an excitability enhancement immediately after stimulation lasting for at least 90 min (Fig. 3*A*). For cathodal tDCS with 2 mA, a gradual increase of facilitation, reaching the peak 120 min after tDCS and returning to baseline values after 6–8 h, can be observed (Fig. 3*B*). In contrast, 1 mA cathodal tDCS resulted in a significant enhancement of intracortical inhibition, and a respective reduction of facilitation (Fig. 3*C*). The effects of 2 mA cathodal tDCS are qualitatively different from those of 1 mA stimulation (Nitsche *et al.* 2005), and are more similar to that of 2 mA anodal stimulation. Thus, it might be speculated that 2 mA anodal and cathodal tDCS have similar mechanisms of action on intracortical systems, which might be mediated by a calcium increase in the LTP range for both stimulation protocols.

No effect on I-wave facilitation and cortical silent period. For the 2 mA stimulation protocols, the results show no effect on either polarity of I-wave facilitation or the cortical silent period. Essentially the same holds true for the effects of 1 mA cathodal tDCS. These missing effects differ from those of previous studies with regard to I-wave facilitation, where 1 mA stimulation had an effect (Nitsche *et al.* 2005; Lang *et al.* 2011). For the 2 mA conditions, the missing effect in the present study might be due to the fact that both parameters were solely obtained immediately after tDCS, when the stimulation might have had only minor effects on cortical excitability, as can be derived from the missing effect on single-pulse MEP amplitudes. Furthermore, in contrast to the above-mentioned TMS protocols, which are influenced by glutamatergic mechanisms, I-wave facilitation and CSP are primarily controlled by the GABAergic system (Paulus *et al.* 2008), on which tDCS might have no major impact (Nitsche *et al.* 2004*b*). At first sight, this seems to contradict the results of a recently published magnetic resonance spectroscopy (MRS) study, which showed a decrease in free GABA concentration within the stimulated area after 10 min of both anodal and cathodal 1 mA tDCS (Stagg *et al.* 2009). Reasons for the opposing results might be the differences of stimulation protocols with regard to stimulation intensity and duration. Furthermore, the amount of free GABA concentration might not translate one to one to TMS-induced activity of GABAergic synapses. Moreover, it cannot be excluded that the longer and stronger protocols in the present study have different effects on GABAergic neurons (e.g. due to depth of the induced electrical field). Finally, CSP and I-wave facilitation were obtained only immediately after stimulation due to temporal restrictions, and at this time point the excitability alterations were not significant as well with regard to other stimulation protocols.

General remarks

Taken together, the results of our study show that the enhancement or prolongation of tDCS intensity or stimulation duration is not always accompanied by an increase of its efficacy, but might even change the direction of effects. This leads to the assumption that in healthy subjects a 'ceiling effect' of single stimulation protocols might exist, which cannot be overcome with simply more intensive stimulation. However, repeated stimulation protocols might be candidates to enhance the efficacy of stimulation (Monte-Silva *et al.* 2010), and also pharmacological interventions have been shown to prolong the after-effects of tDCS for up to about 24 h after the end of stimulation (Nitsche *et al.* 2004*a*; Kuo *et al.* 2008; Monte-Silva *et al.* 2009).

It is not self-evident that the results of this study, which was conducted in healthy young subjects, translate one-to-one to the effects in neuropsychiatric patients. In neuropsychiatric diseases, transmitter availability and other features of brain function might be different, and have a prominent impact on the efficacy of non-invasive brain stimulation to alter cortical excitability. Moreover, in clinical protocols often repetitive stimulation is performed, which might have an impact on the resulting plasticity. Finally, it is not completely clear if the neuroplastic effects of tDCS determine the clinical efficacy in each case. Nevertheless, the results of the present study argue for the importance to probe the physiological effects of extended stimulation protocols, and not to take enhanced efficacy of stronger protocols for granted.

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Author contributions

The experiments were conducted at the University Medical Center, Dept. Clinical Neurophysiology, Georg-August-University, Goettingen. M.A.N., M.-F.K., G.B., V.M., and W.P. contributed to the design and conception of the experiments. G.B., V.M., and M.-F.K., and M.A.N. contributed to the collection, analysis, and interpretation of the data. G.B. drafted the paper, and M.A.N., M.-F.K., V.M., and W.P. revised it critically for important inellectual content. All authors approved the final version of the manuscript.

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2.2. Effect of Serotonin on Paired Associative Stimulation-Induced Plasticity in the Human Motor Cortex

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Effect of Serotonin on Paired Associative Stimulation-Induced Plasticity in the Human Motor Cortex

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Serotonin modulates diverse brain functions. Beyond its clinical antidepressant effects, it improves motor performance, learning and memory formation. These effects might at least be partially caused by the impact of serotonin on neuroplasticity, which is thought to be an important foundation of the respective functions. In principal accordance, selective serotonin reuptake inhibitors enhance long-term potentiation-like plasticity induced by transcranial direct current stimulation (tDCS) in humans. As other neuromodulators have discernable effects on different kinds of plasticity in humans, here we were interested to explore the impact of serotonin on paired associative stimulation (PAS)-induced plasticity, which induces a more focal kind of plasticity, as compared with tDCS, shares some features with spike timing-dependent plasticity, and is thought to be relative closely related to learning processes. In this single-blinded, placebo-controlled, randomized crossover study, we administered a single dose of 20 mg citalopram or placebo medication and applied facilitatory- and excitability-diminishing PAS to the left motor cortex of 14 healthy subjects. Cortico-spinal excitability was explored via single-pulse transcranial magnetic stimulation-elicited MEP amplitudes up to the next evening after plasticity induction. After citalopram administration, inhibitory PAS-induced after-effects were abolished and excitatory PAS-induced after-effects were enhanced trendwise, as compared with the respective placebo conditions. These results show that serotonin modulates PAS-induced neuroplasticity by shifting it into the direction of facilitation, which might help to explain mechanism of positive therapeutic effects of serotonin in learning and medical conditions characterized by enhanced inhibitory or reduced facilitatory plasticity, including depression and stroke.
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Keywords: neuroplasticity; serotonin; paired associative stimulation; human; motor cortex; depression

INTRODUCTION

Serotonin (5-HT) is a widely distributed neurotransmitter in the brains of animals and humans, affecting various physiological functions such as learning, memory formation, pain perception, mood and the sleep–wakefulness cycle (Bert *et al*, 2008; Geyer, 1996; Hasbroucq *et al*, 1997; Jacobs and Fornal, 1997; Meneses, 1999). One important foundation for these effects might be its impact on neuroplasticity. Activation of serotoninergic subreceptors is shown to affect long-term potentiation (LTP) or long-term depression (LTD) in animal slice preparations, depending on subreceptor type, location and frequency of application (Huang and Kandel, 2007; Kojic *et al*, 1997; Mori *et al*, 2001).

In the clinical domain, studies have suggested that depression might be a result of altered brain plasticity (Christoffel *et al*, 2011; Garcia, 2002; Henn and Vollmayr, 2004; Popoli *et al*, 2002), on which serotonin has a major impact. Distress has been proposed as one of the main factors preceding depression (Caspi *et al*, 2003), and in animals it inhibits LTP and facilitates LTD induction (Foy *et al*, 1987; Rocher *et al*, 2004; Shors *et al*, 1989; Xu *et al*, 1997). In accordance, LTD is facilitated in animal models of depression, which was prevented by chronic application of the selective serotonin reuptake inhibitor (SSRI) fluvoxamine (Holderbach *et al*, 2007). Besides depression, several studies have demonstrated that SSRIs improve motor functions in stroke patients (Chollet *et al*, 2011; Dam *et al*, 1996; Pariente *et al*, 2001) and in healthy individuals (Loubinoux *et al*, 1999; Loubinoux *et al*, 2002a; Loubinoux *et al*, 2002b; Loubinoux *et al*, 2005). Again, the physiological basis for this effect might be the impact of serotonin on plasticity.

Recently it was shown that motor cortex plasticity in healthy humans induced by transcranial direct current stimulation (tDCS) was affected by single-dose SSRI. Citalopram enhanced facilitatory plasticity induced by anodal tDCS and converted cathodal tDCS-induced inhibitory plasticity into facilitation (Nitsche *et al*, 2009). tDCS and paired associative stimulation (PAS) are non-invasive brain stimulation techniques inducing changes in cortical excitability that outlast the stimulation duration (Nitsche *et al*, 2003b; Nitsche and Paulus, 2000, 2001; Stefan *et al*, 2000; Wolters *et al*, 2003). These alterations in cortical

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excitability are NMDA- and Ca^{2+}-dependent (Nitsche *et al*, 2003a; Stefan *et al*, 2002; Wolters *et al*, 2003). tDCS induces non-focal plasticity, affecting relatively non-selectively neuronal populations beneath the large stimulation electrodes via subthreshold resting membrane potential modulation (Nitsche *et al*, 2008; Nitsche *et al*, 2007; Purpura and McMurtry, 1965). PAS induces focal and synapse-specific plasticity of the respective target neurons. In PAS, a repetitive electric pulse to a peripheral nerve at an intensity that activates somatosensory afferents is combined with transcranial magnetic stimulation (TMS) over the corresponding area of the primary motor cortex. Depending on the interstimulus interval (ISI), synchronous or asynchronous activation of the target group of neurons, which are motor cortex neurons connected with the respective somatosensory afferents, is accomplished, resulting in excitatory or inhibitory after-effects (Stefan *et al*, 2000). This mechanism of plasticity induction resembles some characteristics of spike timing-dependent plasticity (STDP), which is closely linked to learning and memory processes.

Interestingly, other neuromodulators have discernable effects of tDCS- and PAS-induced plasticity. Specifically, dopamine, acetylcholine, and nicotine have a focusing, or signal-enhancing effect on facilitatory plasticity (Kuo *et al*, 2007; Kuo *et al*, 2008; Monte-Silva *et al*, 2010; Thirugnanasambandam *et al*, 2012). These substances abolish tDCS-induced non-focal, but enhance PAS-generated focal facilitatory plasticity. This effect might explain the cognition- and behavior-enhancing impact of these substances.

After having explored the impact of serotonin on tDCS-induced plasticity, we were now interested to explore how this modulator affects PAS-generated neuroplastic cortical excitability alterations. We hypothesize that citalopram enhances PAS-induced focal excitatory plasticity and abolishes focal inhibitory plasticity or convert it into excitation, as it was shown for tDCS (Nitsche *et al*, 2009).

MATERIALS AND METHODS

Subjects

Fourteen healthy subjects aged 28.1 ± 4.7 years (7 males/7 females) were recruited. All subjects were right-handed according to the Edinburgh handedness inventory (Oldfield, 1971). None of them took any medication, had a history of a neuropsychiatric disease, present pregnancy, or metallic head implants. All volunteers gave written informed consent and were compensated for participation. The investigation was approved by the Ethics Committee of the University of Göttingen, and conforms to the principles laid down in the Declaration of Helsinki.

Paired Associative Stimulation

The peripheral electric pulse was delivered over the right ulnar nerve at the level of the wrist, followed by a TMS pulse over the M1 representation of the abductor digiti minimi muscle (ADM) at ISIs of 10 (PAS10) or 25 ms (PAS25). The peripheral pulse was delivered by a Digitimer D184 multipulse stimulator (Digitimer, Welwyn Garden City, UK) at an intensity of 300% of the sensory perceptual threshold. The TMS pulse was delivered by a Magstim 200 stimulator with an intensity to elicit single-pulse MEPs with peak-to-peak amplitudes of on average 1 mV. The participants were instructed to count silently the number of pulses they received at their wrist during the whole stimulation duration to guarantee sufficient attention to the procedure, which has been shown to be crucial to obtain the intended after-effects (Stefan *et al*, 2000; Stefan *et al*, 2004).

Pharmacological Interventions

Citalopram (20 mg) or equivalent placebo (PLC) drugs were administered 2 h before the start of the experimental session, allowing the verum drug to induce a stable plasma level and produce prominent effects in the central nervous system (Bezchlibnyk-Butler *et al*, 2000; Kragh-Sorensen *et al*, 1981; Robol *et al*, 2004).

Monitoring of Motor Cortical Excitability

MEPs were recorded from the right ADM by single-pulse TMS over the left primary motor cortex, conducted by a Magstim 200 magnetic stimulator (Magstim, Whiteland, Dyfed, UK) with a figure-of-eight magnetic coil (diameter of one winding—70 mm; peak magnetic field—2.2 T). The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline. The optimal coil placement (hotspot) was defined as the site where TMS resulted consistently in the largest MEPs of the contralateral ADM. Surface MEPs were recorded from the right ADM with Ag–AgCl electrodes in a belly-tendon montage. The signals were amplified, and band-pass filtered (2 Hz to 2 kHz, sampling rate, 5 kHz). Signals were digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK), controlled by Signal Software (Cambridge Electronic Design, v. 2.13) and stored for offline analysis.

Experimental Procedures

Each subject participated in four experimental sessions (PAS25 with citalopram or placebo, PAS10 with citalopram or placebo), which were carried out in randomized order and separated by minimum 1 week. A unique sequence of experimental sessions was randomly generated for each subject individually, which did not match any previously generated one for other subjects. The volunteers were seated in a comfortable chair with head and arm rests. First, the hotspot (the position of coil that produced the largest MEPs of the right ADM) was identified by TMS. Then the stimulation intensity was adjusted to elicit single-pulse MEPs with peak-to-peak amplitudes of on average 1 mV and 25 MEPs were recorded for the first baseline determination. After baseline recording, citalopram or placebo medication was administered. At 2 h after intake of medication, a second baseline was recorded to monitor for a possible influence of the drug on cortical excitability (baseline 2), and TMS intensity was adjusted, if necessary (baseline 3). After that procedure, PAS25 or PAS10 was administered and 25 MEPs were recorded immediately after stimulation and at time points of 5, 10, 15, 20, 25, 30, 60, 90 and 120 min after the stimulation PAS. Further TMS measurements were

conducted in the evening of the same day (SE), next morning, at ~0900 hours (NM), next noon, at ~1200 hours (NN), and next evening, at ~1800 hours (NE) (Figure 1). To keep the EMG electrodes and TMS coil at the same place for later measurements, their positions were marked with a waterproof pen. Subjects were blinded for both, stimulation and medication conditions; the experimenter was blinded only for the medication condition.

Analysis and Statistics

The experimenter was unblinded after finishing data collection and analysis. The individual means of 25 MEP amplitudes were calculated for all subjects and the after-stimulation mean MEP amplitudes were normalized to the respective mean baseline MEP amplitudes (quotient of post-PAS MEPs *vs* baseline values). Then the grand averages for each time point were calculated. A repeated measures ANOVA was performed on the above-mentioned data using MEP amplitude as the dependent variable and medication, stimulation type and time course as within-subject factors. The Mauchly test of sphericity was performed and the Greenhouse–Geisser correction applied when necessary. In case of significant results of the ANOVA, exploratory *post-hoc* comparisons were performed using Student's *t*-tests (paired samples, two-tailed, $p<0.05$, not corrected for multiple comparisons) between the MEP amplitudes before and after PAS administration within one experimental condition and between the single time points within the same stimulation condition.

To exclude differences between baseline values of different conditions, also between first and second baseline values, we compared the respective values by Student's *t*-tests (paired samples, two-tailed, $p<0.05$, not corrected for multiple comparisons).

RESULTS

All subjects tolerated the procedure well. None of them reported any side effect of either citalopram or the stimulation.

The average baseline MEP values did not significantly differ between groups as revealed by Student's *t*-tests (paired samples, two-tailed, $p>0.05$). Citalopram alone did not have any impact on cortical excitability, as revealed by Student's *t*-tests between first and second baseline values (paired samples, two-tailed, $p>0.05$; Table 1).

The ANOVA revealed significant main effects of medication ($F(1)=5.345$; $p=0.039$), stimulation ($F(1)=39.497$; $p<0.001$), stimulation × time ($F(14)=15.593$; $p<0.001$) and medication × time ($F(14)=2.456$; $p=0.004$) interactions (for details, see Table 2). The main effect of medication is caused by similarly directed effects of citalopram on MEP amplitudes for PAS10, and PAS25. As compared with placebo medication, citalopram enhanced motor cortical excitability. The main effect of stimulation is due to relatively larger MEP amplitudes in the PAS25 condition, as compared with PAS10, irrespective of medication condition or time point. The interaction of stimulation x time refers to different time courses of MEP alterations generated by PAS10, and PAS25. MEP reductions induced by PAS10 lasted longer than those accomplished by PAS25, and were antagonistically directed for up to 90 min after stimulation, but not with regard to the later time points. Finally, the interaction of medication and time course is caused by the MEP-enhancing effect of citalopram on MEP amplitudes, as compared with placebo medication, during the first 30 (PAS25) or 90 (PAS10) min after PAS, but not for later time points.

Post-hoc Student's *t*-tests show that in the placebo medication conditions, MEPs were significantly enhanced for 30 min after PAS-25 stimulation and diminished for 90 min after PAS10 stimulation. Citalopram abolished PAS10-induced LTD-like plasticity and enhanced PAS25-induced LTP-like plasticity, as compared with the respective placebo medication conditions. Student's *t*-tests show significant differences between drug and placebo conditions at all time points between 0 and 25 min after PAS10 administration and only at the single time point of 30 min after PAS25 (Figure 2).

For the effects of citalopram on PAS-induced plasticity with regard to the grand average calculated for the first 30 min after PAS, citalopram had a significant effect on focal

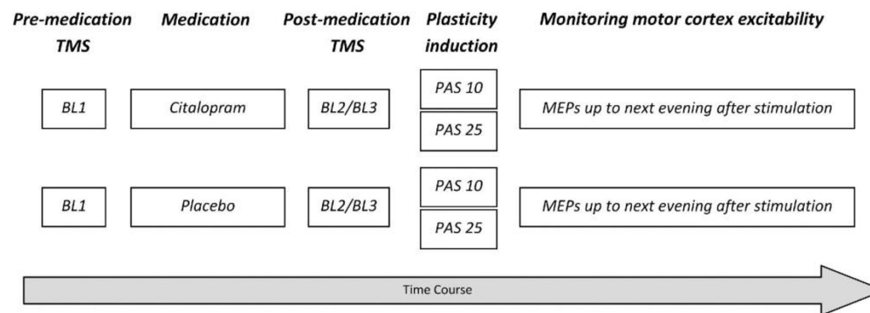

Figure 1 Course of the study. In the beginning of each session, before administration of citalopram or placebo medication, 25 baseline single-pulse MEPs were recorded at an intensity to elicit MEPs with peak-to-peak amplitudes of on average 1 mV. After 2 h, the second baseline was recorded to explore the effect of medication on cortical plasticity, and adjusted, if necessary. After obtaining the second (or third) baseline, PAS was administered and 25 MEPs were recorded immediately after stimulation and at time points of 5, 10, 15, 20, 25, 30, 60, 90, and 120 min after plasticity induction. Further transcranial magnetic stimulation (TMS) measurements were conducted in the evening of the same day (SE), next morning, at ~0900 hours (NM), next noon, at ~1200 hours (NN), and next evening, at ~1800 hours (NE).

Table 1 MEP Amplitudes and Stimulation Intensity Before and After Citalopram Administration

Stimulation	TMS parameter	Medication condition	Baseline 1	Baseline 2	Baseline 3
PAS25	MEP	Citalopram	1.04 ± 0.07	0.99 ± 0.17	0.99 ± 0.14
		Placebo	1.03 ± 0.11	0.93 ± 0.23	0.96 ± 0.14
	%MSO	Citalopram	49.3 ± 9.81	49.1 ± 9.85	49.3 ± 9.98
		Placebo	48.9 ± 9.42	48.9 ± 9.42	49.3 ± 9.46
PAS10	MEP	Citalopram	1.04 ± 0.12	1.00 ± 0.12	1.00 ± 0.10
		Placebo	1.04 ± 0.09	0.95 ± 0.11	1.03 ± 0.10
	%MSO	Citalopram	49.4 ± 9.48	49.4 ± 9.48	49.4 ± 9.53
		Placebo	49.1 ± 9.61	49.1 ± 9.61	49.6 ± 9.80

Shown are the mean MEP amplitudes ± SD and stimulation intensity (percentage of maximum stimulator output, %MSO) mean ± SD of baselines 1, 2 and 3. The intensity of TMS was adjusted to elicit MEPs with peak-to-peak amplitude of ~1 mV (baseline 1). A second baseline (baseline 2) was recorded 2 h after citalopram or placebo intake to determine the impact of the drug on cortical excitability and adjusted if necessary (baseline 3). Student's *t*-tests revealed no significant differences between conditions ($p > 0.05$).

Table 2 Results of the Repeated Measures ANOVA

Factor	Df	*F*	*p*
Medication	1	5.345	0.039*
Stimulation	1	39.497	<0.001*
Time	14	0.723	0.749
Medication × stimulation	1	0.543	0.476
Medication × time	14	2.456	0.004*
Stimulation × time	14	15.593	<0.001*
Medication × stimulation × time	14	0.622	0.845

*Significant results at $p < 0.05$.

excitability-diminishing plasticity, as revealed by respective *post-hoc* Student's *t*-tests (Student's *t*-test, paired samples, two-tailed, $p = 0.009$), whereas only a non-significant tendency towards excitability enhancement after PAS25 stimulation was detected (Student's *t*-test, paired samples, two-tailed, $p = 0.126$) (Figure 3).

DISCUSSION

The results of this study show that serotonin has specific effects on PAS-induced motor cortex plasticity in healthy humans. It abolishes focal LTD-like and trendwise enhances focal LTP-like plasticity induced by PAS10 and PAS25, respectively.

These results go in line with previous studies (Nitsche *et al*, 2009; Normann *et al*, 2007). Chronic application of SSRI enhanced facilitatory plasticity and resulted in a shift of inhibitory plasticity of early visual-evoked potentials (VEPs) toward excitation (Normann *et al*, 2007) or restored LTP induction and suppressed LTD facilitation in distressed animals (Von Frijtag *et al*, 2001). For the human motor cortex, another study has demonstrated that a single dose of the SSRI citalopram results in enhancement and prolongation of anodal tDCS-induced LTP-like facilitation and conversion of cathodal tDCS-induced LTD-like plasticity into facilitation (Nitsche *et al*, 2009). In further accordance,

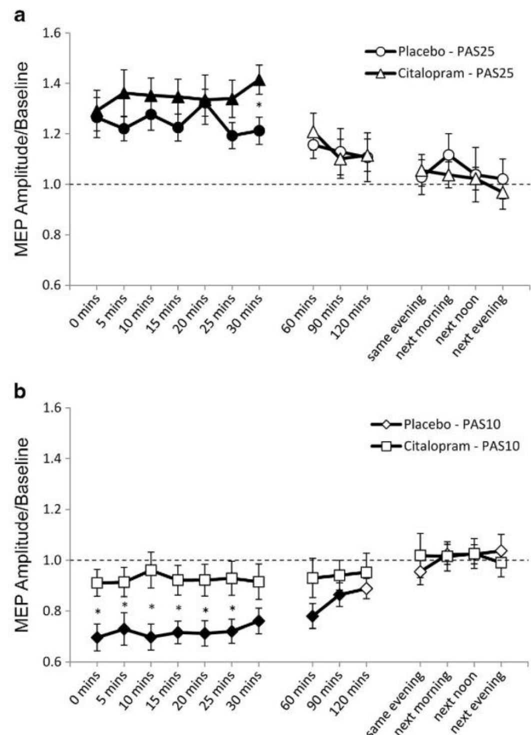

Figure 2 Impact of citalopram on paired associative stimulation (PAS)-induced neuroplasticity. Shown are baseline-normalized MEP amplitudes after plasticity induction by PAS25 (a) and PAS10 (b) under placebo or citalopram medication conditions up to the evening of the post-stimulation day. (a) In the placebo medication condition, PAS25 induced a significant excitability elevation up to 60 min after stimulation, which was enhanced, but not prolonged, by citalopram. (b). In the placebo medication condition, cortical excitability was significantly reduced after PAS10, this effect was abolished by citalopram. Error bars indicate SEM. Filled symbols indicate significant differences of post-stimulation MEP amplitudes from respective baseline values; asterisks indicate significant differences between the drug and placebo medication conditions at the same time points (Student's *t*-test, two-tailed, paired samples, $p < 0.05$).

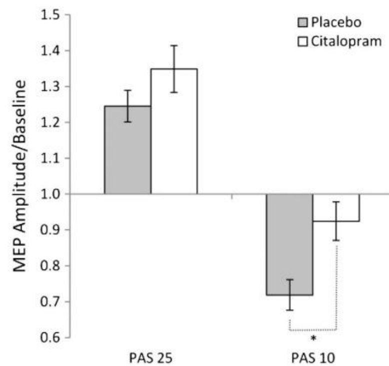

Figure 3 Citalopram enhances PAS25-induced excitatory plasticity and abolishes PAS10-induced inhibitory plasticity. Each column represents the mean of baseline-normalized MEP ± SEM amplitudes until 30 min after stimulation. Asterisks indicate significant differences between drug and placebo conditions (Student's *t*-test, two-tailed, paired samples, $p < 0.05$).

animal studies have shown that serotonin can enhance LTP (Huang and Kandel, 2007; Kojic *et al*, 1997; Machacek *et al*, 2001; Mori *et al*, 2001; Ohashi *et al*, 2002; Park *et al*, 2012), block the stress-caused inhibition of LTP (Ryan *et al*, 2008) or block LTD (Normann *et al*, 2007). Activation of 5-HT receptors was furthermore shown to reverse LTD induction or convert it into LTP (Costa *et al*, 2012; Kemp and Manahan-Vaughan, 2005).

However, activation of serotoninergic receptors also had opposing results on plasticity in other studies. Some studies have shown negative or no effect of serotonin on LTP induction (Edagawa *et al*, 1998; Huang and Kandel, 2007; Kojima *et al*, 2003; Normann *et al*, 2007; Sanberg *et al*, 2006), which can be explained by activation of different serotoninergic receptor subtypes, stage of brain development or dosage and frequency of application of 5-HT agonists or antagonists (Mori *et al*, 2001; Park *et al*, 2012; Staubli and Otaky, 1994). To explore the reasons of such opposing results, future studies should address specific serotoninergic receptor subtypes, using different 5-HT-receptor agonist or antagonist drugs. It might also make sense to explore different dosages of serotoninergic receptor agonists, as it has been demonstrated that other neuromodulators, such as dopamine, have dose-dependent effects on focal and non-focal plasticity in humans (Monte-Silva *et al*, 2009; Monte-Silva *et al*, 2010).

Proposed Mechanisms of Action

After-effects of tDCS and PAS are NMDA- and Ca^{2+}-dependent (Nitsche *et al*, 2003a; Stefan *et al*, 2002; Wolters *et al*, 2003). It has been shown that serotonin facilitates NMDA receptor-dependent LTP (Park *et al*, 2012). Furthermore, serotonin affects K^{+}-channels and reduces membrane potassium conductance (Andrade and Chaput, 1991; Bockaert *et al*, 1992; Choi and Hahn, 2012; Jeong *et al*, 2012; Panicker *et al*, 1991). In case of enhanced serotonin level, these factors could result in membrane depolarization and enhanced Ca^{2+} influx into the postsynaptic neurons through calcium channels and NMDA receptors (Gu, 2002). The direction of induced plasticity depends on the amount of intracellular calcium, with low concentration inducing LTD, high concentration inducing LTP and medium concentration resulting in no plasticity (Cho *et al*, 2001; Lisman, 2001). Therefore, the above-mentioned serotonin-triggered enhancement of calcium influx could have resulted in a tendency towards facilitation of PAS25-induced LTP-like plasticity, similar to that accomplished by anodal tDCS in a previous study (Nitsche *et al*, 2009). Unlike for cathodal tDCS, where neuroplastic excitability diminutions were converted to facilitation by citalopram (Nitsche *et al*, 2009), the drug abolished PAS10-induced LTD-like plasticity in the present study. This can be explained by differences of the respective plasticity induction protocols. Plasticity induced by tDCS is accomplished by long, tonic depolarization of large neuronal populations and activation of voltage-dependent calcium channels, whereas depolarization caused by PAS is short-lasting and affects only small groups of neurons. Therefore the increase of intracellular calcium might be smaller after PAS administration, as compared with tDCS. Given the dependency of plasticity induction from intracellular calcium level, thus the calcium increase accomplished by citalopram might have been sufficient to induce LTP-like plasticity in case of cathodal tDCS, but not for PAS10. This also explains why the shift in excitability toward PAS25-induced excitatory plasticity enhancement is not as clear as in case of anodal tDCS. This hypothesis should however be tested more directly in future experiments.

The role of specific 5-HT receptors in the impact of citalopram on PAS-generated plasticity is not clear. 5-HT2 and 5-HT3 are candidate receptors. The 5-HT3 receptor enhances Ca^{2+} conductance, leading to neuronal depolarization, while the 5-HT2 receptor induces Ca^{2+} release from intracellular stores (Reiser *et al*, 1989). Accordingly, activation of 5-HT2 receptors has a facilitatory effect on NMDA receptor-dependent LTP induction in the visual cortex of adult rats (Park *et al*, 2012). Finally, serotonin affects cholinergic (Consolo *et al*, 1994; Matsumoto *et al*, 2001; Yamaguchi *et al*, 1997), GABAergic (Roerig and Katz, 1997; Waider *et al*, 2012), nicotinergic (Zaniewska *et al*, 2009), and dopaminergic (Gobert and Millan, 1999; Wood and Wren, 2008) systems, which have a major impact on stimulation-induced plasticity in humans (Kuo *et al*, 2007; Kuo *et al*, 2008; Monte-Silva *et al*, 2009; Monte-Silva *et al*, 2010; Nitsche *et al*, 2004; Thirugnanasambandam *et al*, 2012). While it cannot be ruled out completely that serotonin enhancement affected plasticity partially by its impact on one of these neuromodulatory systems, a profound contribution seems unlikely, because the impact of citalopram on tDCS-, and PAS-induced plasticity differs relevantly from those of other neuromodulators. Specifically the above-mentioned studies show that dopamine, acetylcholine, and nicotine have a focusing effect on LTP-like motor cortex plasticity, which is hypothesized to be advantageous for task performance if stable information processing is needed (eg, a simple task which requires uniform action). In contrast, de-focusing—as obtained by citalopram, which enhances focal and non-focal LTP-like plasticity, as shown in the present study, and in a previous study of our group (Nitsche *et al*, 2009) might be advantageous when a task requires flexible information

processing (eg, complex problem solving) (Seamans and Yang, 2004). This hypothetical specific impact of serotonin on ask performance should be explored in future experiments.

General Remarks

PAS is assumed to be related to learning processes as it shares some characteristics with STDP, such as timing and synchronization of two pulses as a requirement to induce plasticity. Therefore, the results of the present and other studies, which show an enhancement of LTP-like PAS-induced plasticity, and a reduction of LTD-like plasticity by SSRIs (Nitsche *et al*, 2009; Normann *et al*, 2007), make these drugs interesting substances for improving learning and motor performance in several clinical conditions (eg, in motor or speech rehabilitation after stroke). Especially with regard to stroke and depression, where LTP-like plasticity seems to be reduced, and/or LTD-like plasticity enhanced by disease-related processes (Foy *et al*, 1987; Schaechter, 2004; Traversa *et al*, 1997; Traversa *et al*, 1998; Turton *et al*, 1996; Xu *et al*, 1997), the results of the present study can at least partially explain why SSRIs can reduce symptoms. In accordance with the LTP-enhancing effect of SSRI with regard to stimulation-induced plasticity (Nitsche *et al*, 2009), a synergistic effect of tDCS and SSRI medication on major depression has been described recently, most probably related to the increased efficacy of anodal tDCS-induced LTP-like plasticity under SSRI (Brunoni *et al*, 2013).

One possible limitation to our study could be that 1-week intersession interval might not be sufficient to rule out any interference effects definitely, as suggested by the results of a recent study (Rajji *et al*, 2011), where PAS25 and PAS10 had a significant impact on motor task performance 1 week after PAS administration. In our study, MEP amplitudes recorded the day after plasticity induction however show no effect of PAS with or without citalopram on motor cortex excitability. Moreover, because of the randomized order of conditions, we would not expect a systematic impact of any minor carryover effect on the results. Finally, previous studies of our group in which a similar procedure was performed showed PAS plasticity effects in the placebo medication conditions, which are comparable to the experiments of other groups, in which not such a frequent repetition of sessions was performed (Kuo *et al*, 2008; Monte-Silva *et al*, 2009; Monte-Silva *et al*, 2010; Stefan *et al*, 2000; Stefan *et al*, 2004; Thirugnanasambandam *et al*, 2012). Therefore, late-phase plasticity is unlikely to have compromised the results of the present experiments.

Interestingly, chronic application of SSRI has different effects on cortical excitability as compared wth single-dose application, although both conditions resulted in functional improvement of motor performance (Gerdelat-Mas *et al*, 2005; Loubinoux *et al*, 2002a; Loubinoux *et al*, 2002b). Clinical studies show that it takes several weeks to obtain therapeutic effects of SSRIs. This suggests an involvement of different mechanisms, such as desensitization and down-regulation of receptors, or reduction of serotonin synthesis in the effects of chronic administration of SSRIs (Blier and Bouchard, 1994; Pineyro *et al*, 1994; Yamane *et al*, 2001), which should be explored in larger detail in future studies.

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2.3. Effect of the nicotinic $\alpha_4\beta_2$-receptor partial agonist varenicline on non-invasive brain stimulation-induced neuroplasticity in the human motor cortex

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Running title: Impact of varenicline on neuroplasticity

Abstract

The neuromodulator nicotine alters cognitive functions in animals and humans most likely by modification of brain plasticity. In the human brain, it alters plasticity induced by transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS), probably by interference with calcium-dependent modulation of the glutamatergic system. We aimed to test this hypothesis further by exploring the impact of the $\alpha_4\beta_2$ nicotinic receptor partial agonist varenicline, which has calcium channel properties, on focal and non-focal plasticity, induced by PAS and tDCS, respectively. We administered low (0.1mg), medium (0.3mg) and high (1.0mg) single doses of varenicline or placebo medication before PAS or tDCS on the left motor cortex of 25 healthy non-smoking individuals. Corticospinal excitability was monitored by single-pulse transcranial magnetic stimulation (TMS)-induced motor evoked potential (MEP) amplitudes up to 36 hours after plasticity induction. Whereas low-dose varenicline had no impact on stimulation-induced neuroplasticity, medium-dose varenicline preserved only focal excitatory plasticity. High-dose application preserved cathodal tDCS-induced excitability diminution and focal facilitatory plasticity induced by excitatory PAS, but abolished anodal tDCS- and inhibitory

PAS-induced changes in excitability. These results are comparable to the impact of nicotine receptor activation and might help to further explain the involvement of specific receptor subtypes in the nicotinic impact on neuroplasticity and cognitive functions in humans.

Key words: Neuroplasticity; nicotine; varenicline; transcranial direct current stimulation; human; motor cortex.

Introduction

Smoking tobacco is one of the leading risks to human health (Peto et al., 1992, Doll et al., 2005). Nicotine is the main neuroactive component of tobacco responsible for physical dependence and addiction. Besides addictive properties, many studies demonstrate positive effects on cognition. Human and animal studies have shown that nicotine improves attention, motor functions, working and episodic memories (Provost and Woodward, 1991, Hahn et al., 2002, Hahn and Stolerman, 2002, Kumari et al., 2003, Jubelt et al., 2008, Froeliger et al., 2009, Heishman et al., 2010, Mocking et al., 2012). Nicotine also improves learning, attention and perception in patients suffering from Alzheimer's disease (Jones et al., 1992, Wilson et al., 1995, White and Levin, 1999). Nicotine withdrawal is often associated with impairments of working and verbal memory and neuroplasticity, while nicotine re-administration restitutes these functions in smoking individuals (Jacobsen et al., 2005, Cole et al., 2010, Grundey et al., 2012a).

The neurophysiological basis for the nicotinic effects on cognition is hypothesized to be its impact on cortical excitability and plasticity, controlled by activation of $\alpha_4\beta_2$ and α_7 nicotinic acetylcholine (nAChR) receptors. These are ligand-gated ion channels (Burnashev, 1998, Dajas-Bailador and Wonnacott, 2004), which modulate the permeability of Ca^{2+} ions and are centrally involved in plasticity induction (Lisman, 2001). In accordance, animal studies have demonstrated that activation of nicotinic receptors results in LTP facilitation (Matsuyama et al., 2000, Fujii and Sumikawa, 2001a, Welsby et al., 2006, Nakauchi et al., 2007), reversal of GABAergic inhibition of LTP (Fujii et al., 2000) as well as LTD enhancement (Fujii and Sumikawa, 2001b, Ge and Dani, 2005).

Recently, studies in humans demonstrated that global cholinergic activation increases focally, but abolishes non-focally induced LTP-like plasticity, whereas it preserves and prolongs both focal and non-focal LTD-like plasticity. For nicotine, a similar effect was seen for LTP-like plasticity, but LTD-like plasticity was abolished by this substance in non-smoking healthy humans (Kuo et al., 2007, Thirugnanasambandam et al., 2012). These results show a partial dissociation of the impact of global cholinergic activation and nicotinic receptor activation on plasticity. Furthermore, the "focusing effect" on LTP-like plasticity might explain a beneficial impact on cognition.

In these studies, focal and non-focal plasticity was induced by transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS), respectively. Both, tDCS and PAS are non-invasive brain stimulation techniques inducing long-lasting changes of cortical excitability which are Ca^{2+} and NMDA receptor-dependent (Nitsche and Paulus, 2000, Stefan et al., 2000, Nitsche and Paulus, 2001, Stefan et al., 2002, Nitsche et al., 2003a, Nitsche et al., 2003b, Wolters et al., 2003). Neuroplastic changes induced by tDCS are non-focal and affect neuronal populations beneath the relatively large stimulation electrodes via subthreshold resting membrane potential modulation (Purpura and McMurtry, 1965, Nitsche et al., 2007, Nitsche et al., 2008). In contrast, plasticity induced by PAS is presumed to be focal, synapse-specific and timing-dependent, affecting only selective neuronal populations. During PAS, a repetitive electric pulse to a peripheral nerve is combined with suprathreshold transcranial magnetic stimulation (TMS)-pulse over the corresponding area of the primary motor cortex. The target group of somatosensory-motor cortical synaptic connections, is activated synchronously or asynchronously by combined peripheral and TMS pulses, depending on the interstimulus interval (ISI), resulting in excitatory or inhibitory after-effects (Stefan et al., 2000). PAS is thought to be closely linked to learning and memory processes, as its mechanism resembles some characteristics of spike timing-dependent plasticity (STDP) (Stefan et al., 2002, Wolters et al., 2003, Caporale and Dan, 2008).

Beyond unspecific activation of nicotinic receptors by nicotine, not much is known about the contribution of nicotinic receptor subtypes on neuroplasticity in humans. Given that tDCS and PAS induce calcium-dependent plasticity, it can be speculated that specifically nicotinic receptors with calcium channel properties might be involved. In the present study, we aimed to explore

the contribution of $\alpha_4\beta_2$ receptors on non-invasive brain stimulation-induced focal and non-focal plasticity in human non-smokers via application of varenicline. Varenicline is an effective smoking cessation agent (Coe et al., 2005), which is a high-affinity partial agonist to $\alpha_4\beta_2$ and full agonist to α_7 nAChRs (Mihalak et al., 2006). We hypothesized that effective dosages of the drug should, similar to the effect of nicotine (Thirugnanasambandam et al., 2012), abolish tDCS-induced non-focal plasticity and preserve PAS-induced focal excitatory plasticity in non-smoking healthy subjects.

Materials and methods

Subjects

Twenty-five healthy non-smoker subjects aged 24.8 ± 4.4 years (11 males/15 females) were recruited. Two subjects did not finish the experiment. All subjects were right-handed according to the Edinburgh handedness inventory (Oldfield, 1971). None of them took any medication, had a history of a neuropsychiatric or medical disease, present pregnancy, or metallic head implants. All volunteers gave written informed consent and were compensated for participation. The investigation was approved by the Ethics Committee of the University of Göttingen, and conforms to the principles laid down in the Declaration of Helsinki.

Transcranial Direct Current Stimulation

Twelve subjects aged 24.4 ± 4.7 years (4 males/8 females) participated in tDCS experiment. Direct current was delivered by a battery-driven constant current stimulator (neuroConn GmbH, Ilmenau, Germany) through a pair of rubber electrodes covered with saline-soaked sponges (5 x 7 cm). The motor cortex electrode was fixed over the area representing the right abductor digiti minimi muscle (ADM) and the return electrode contralaterally above the right supraorbital area. Subjects received 1mA of either excitability-enhancing anodal tDCS for 13 minutes or excitability-

diminishing cathodal tDCS for 9 minutes, which induces motor cortex excitability alterations lasting for about 1 h (Nitsche and Paulus, 2001, Nitsche et al., 2003b).

Paired Associative Stimulation

Twelve subjects 25 ± 4.4 years (6 males/6 females) participated in PAS experiment. The peripheral electric pulse over the right ulnar nerve at the level of the wrist at an intensity of 300% of the sensory perceptual threshold was followed by a TMS pulse over the M1 representation of the abductor digiti minimi muscle (ADM) at ISIs of 10ms (PAS10) or 25ms (PAS25) at a frequency of 0.05Hz. The peripheral electric pulse was delivered by a Digitimer D184 multipulse stimulator (Digitimer, Welwyn Garden City, United Kingdom). The TMS pulse was delivered by a Magstim 200 stimulator with an intensity to elicit single pulse MEPs with peak-to-peak amplitudes of on average 1 mV. The participants were instructed to silently count the number of pulses they received at their wrist during the whole stimulation duration to guarantee sufficient attention to the procedure, which has been shown to be crucial to obtain the desired after-effects (Stefan et al., 2000, Stefan et al., 2004).

Pharmacological Interventions

Low (0.1mg), medium (0.3mg) or high (1.0mg) dosages of varenicline or 0.5 mg placebo were administered in form of two-piece gelatin capsules (size 2, 18mm length, 6.35mm external diameter) 3 hours before the start of the experimental session, allowing the verum drug to induce a maximum plasma level and produce prominent effects in the central nervous system (Faessel et al., 2006, Obach et al., 2006, Faessel et al., 2010).

Monitoring of motor cortical excitability

MEPs were recorded from the right ADM by single-pulse TMS over the corresponding left primary motor cortex, conducted by a Magstim 200 magnetic stimulator (Magstim, Whiteland,

Dyfed, United Kingdom) with a figure-of-eight magnetic coil (diameter of one winding – 70mm; peak magnetic field - 2.2 T). The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45^0 from the midline. The hotspot was defined as the optimal coil placement, where the TMS pulse resulted consistently in the largest MEPs of the contralateral ADM. Surface MEPs were recorded from the right ADM with Ag-AgCl electrodes in a belly-tendon montage. The signals were amplified, and band-pass filtered (2Hz to 2kHz, sampling rate, 5kHz), digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK), controlled by Signal Software (Cambridge Electronic Design, v. 2.13), and stored for offline analysis.

Experimental procedures

A unique sequence of experimental sessions was randomly generated individually for each subject, which did not match any previously generated one for other subjects. The participants were seated in a comfortable chair with head and arm rests. In the beginning, the hotspot was identified by TMS and then the stimulation intensity was adjusted to elicit single pulse MEPs with peak-to-peak amplitudes of on average 1 mV. Then twenty-five MEPs were recorded for the determination of first baseline. After baseline recording, varenicline or placebo medication was administered. Three hours after intake of medication, a second baseline was recorded to monitor for a possible impact of the drug alone on cortical excitability (baseline 2), and TMS intensity was adjusted, if necessary (baseline 3). After that procedure, the respective plasticity induction protocol was administered (cathodal tDCS, anodal tDCS, PAS10 or PAS25) and twenty-five MEPs were recorded at time points of 0, 5, 10, 15, 20, 25, 30, 60, 90 and 120 minutes after tDCS. Further TMS measurements were conducted in the evening of the same day (SE), next morning, at ~9:00 AM (NM), next noon, at ~12:00PM (NN) and next evening, at ~6:00PM (NE) (Figure 1). To keep the EMG electrodes and TMS coil at the same place for later measurements, their positions were marked with a waterproof pen. The minimum period between two consecutive experimental sessions for a single subject was seven days. Subjects were blinded for both, stimulation and medication conditions; the experimenter was blinded for the medication condition.

Analysis and statistics

The experimenter was unblinded after finishing data collection and analysis. The individual means of 25 MEP amplitudes were calculated at each time point for every subject and the post-tDCS mean MEP amplitudes were normalized to the respective mean baseline MEP amplitudes (quotient of post-stimulation MEPs vs pre-stimulation values: baseline 2, or, if TMS intensity had to be adjusted, baseline 3). Then the grand averages for each time point were calculated. A repeated measures ANOVA was performed on the above-mentioned data separately for tDCS and PAS experiments, using MEP amplitude as the dependent variable and medication, stimulation type and time course as within-subject factors. The Mauchly test of sphericity was performed and the Greenhouse-Geisser correction applied when necessary. In case of significant results of the ANOVA, exploratory post hoc comparisons were performed using Student's t tests (paired samples, two-tailed, $p < 0.05$, not corrected for multiple comparisons) between the MEP amplitudes before and after intervention within one experimental condition and between the single time points (medication vs placebo) within the same stimulation condition.

To compare main effects of different dosages of varenicline on plasticity, averaged MEPs for the first 30 minutes after stimulation were calculated for each subject per experimental session and normalized to baseline 2 (or baseline 3, if TMS intensity was adjusted). Then, these averaged MEP values for each dosage condition were compared with the respective placebo condition by Student's t-tests (paired samples, two-tailed, $p < 0.05$, not corrected for multiple comparisons).

To exclude differences between baseline values of different conditions, and also between first, second and third baseline values, the respective values were compared using Student's t-tests (paired samples, two-tailed, $p < 0.05$, not corrected for multiple comparisons).

Results

All subjects tolerated the procedure well. Only five of them reported slight dizziness, lasting for about one hour after drug intake, which is a mild side effect of varenicline.

Two participants (one from tDCS and one from PAS experiment) left the study after first experimental day due to time constraints.

The average baseline MEP values did not significantly differ between groups as revealed by Student's t tests (paired samples, two-tailed, $p > 0.05$). Varenicline alone did not have any impact on cortical excitability at any dosage, as revealed by Student's t tests between first, second, and third baseline values (paired samples, two-tailed, $p > 0.05$) (Table 1).

Effect of varenicline on tDCS-induced plasticity

The ANOVA revealed significant main effects of STIMULATION ($F(1)=117.900$; $p<0.001$), MEDICATION x STIMULATION ($F(3)=5.050$; $p=0.005$), STIMULATION x TIME ($F(14)=10.013$; $p<0.001$) and MEDICATION x STIMULATION x TIME ($F(42)=2.375$; $p<0.001$) interactions (for details see table 2).

Post-hoc Student's t tests show that in the placebo and low dose varenicline medication conditions, MEPs were significantly enhanced for 60 minutes after anodal and reduced after cathodal tDCS as compared to respective baseline values. MEPs obtained under low-dose varenicline did not differ from those under placebo medication at any time point. Medium dose varenicline abolished both anodal and cathodal tDCS-induced after effects. Here MEP amplitudes did not differ from baseline values at any time point, and MEPs were significantly altered as compared to the respective placebo medication conditions for up to 30 min after tDCS. Under high-dose varenicline, the cathodal tDCS-induced excitability diminution was significant versus baseline until the evening after tDCS, but did not differ significantly from the placebo medication condition. For anodal tDCS, the respective excitability enhancement was initially abolished, and MEPs were significantly smaller than those under placebo medication for the first 10 min after tDCS. However, MEPs were enhanced versus baseline between 25 and 30 minutes after plasticity induction (Figure 2 A,B).

For the effects of different dosages of varenicline on tDCS-induced plasticity with regard to the grand average calculated for the first 30 min after intervention, medium dose of varenicline had a significant abolishing effect on both excitability-enhancing and -diminishing non-focal

plasticity, as revealed by respective student's t-tests (Student's t test, paired samples, two-tailed, $p<0.01$). Furthermore, the anodal tDCS-induced excitability enhancement was abolished by high dose varenicline (Student's t test, paired samples, two-tailed, $p=0.02$). Low dose of varenicline showed no significant differences from the respective placebo medication conditions (Student's t test, paired samples, two-tailed, $p > 0.05$) (Figure 4).

Effect of varenicline on PAS-induced plasticity

The ANOVA revealed significant main effects of STIMULATION ($F(1)=19.134$; $p=0.003$), STIMULATION x TIME ($F(14)=19.064$; $p<0.001$) and MEDICATION x STIMULATION x TIME ($F(42)=1.476$; $p=0.035$) interactions (table 2).

Post-hoc Student's t tests show that MEPs were significantly enhanced for about an hour after PAS25 in all medication conditions, and reduced after PAS10 in placebo and low dose varenicline condition as compared to respective baseline values. Medium and high doses of varenicline abolished PAS10-induced after effects. Here MEP amplitudes did not differ from baseline values at any time point, and MEPs were significantly altered as compared to the respective placebo medication conditions for up to 60 min after PAS administration. In all other conditions, MEPs obtained after varenicline administration did not differ from those under placebo medication at any time point (Figure 3 A,B).

For the effects of different dosages of varenicline on PAS-induced plasticity with regard to the grand average calculated for the first 30 min after intervention, medium and high doses of varenicline have a significant abolishing effect on PAS10-induced focal inhibitory plasticity as revealed by the respective student's t-tests (Student's t test, paired samples, two-tailed, $p<0.001$ and $p=0.01$, respectively). The other conditions showed no significant differences from the respective placebo medication conditions (Student's t test, paired samples, two-tailed, $p>0.05$) (Figure 4).

Discussion

The results of this study show that activation of nicotinic $\alpha_4\beta_2$ and possibly, α_7 receptors has specific and dosage-dependent effects on neuroplasticity in healthy human non-smoking individuals. Low-dosage varenicline did not affect plasticity. In contrast, medium dose of the drug preserved only focal LTP-like plasticity. Under high dosages of the drug, non-focal LTD-like and focal LTP-like effects were preserved, but non-focal LTP-like and focal LTD-like plasticity were compromised. The results obtained under medium-dosage varenicline are fairly identical to those of a previous study, which explored the impact of nicotine on tDCS-induced plasticity (Kuo et al., 2007, Thirugnanasambandam et al., 2012). Therefore, we presume that the focusing effect of nicotine on facilitatory plasticity is at least partially caused by $\alpha_4\beta_2$ receptors. As the MEP amplitudes alone were not affected by any dose of varenicline, a direct influence of the drug on cortical excitability can be ruled out.

Proposed Mechanisms of Action

After-effects of tDCS and PAS are NMDA receptor- and Ca^{2+}-dependent (Stefan et al., 2002, Nitsche et al., 2003a, Wolters et al., 2003). Since $\alpha_4\beta_2$ and α_7 nAChRs are ligand-gated ion channels (Burnashev, 1998, Dajas-Bailador and Wonnacott, 2004), they might affect LTP and LTD induction by an alteration of membrane permeability to Ca^{2+} ions (Lisman, 2001). Indeed, in animal slice experiments, agonists of the respective receptors have a prominent impact on stimulation-induced plasticity. Nicotine has been shown to enhance LTP by postsynaptically activating α_7 nicotinic receptors in the rat dentate gyrus (Welsby et al., 2006), and facilitates NMDA-dependent LTP induction (Yamazaki et al., 2005, Yamazaki et al., 2006a, Yamazaki et al., 2006b, Griguoli et al., 2013, Prestori et al., 2013). In another study, activation of both, $\alpha_4\beta_2$ and α_7 nicotinic receptors was essential for LTP induction (Matsuyama and Matsumoto, 2003). Since activation of nAChRs increased intracellular Ca^{2+} in several studies (Chavez-Noriega et al., 1997, Chavez-Noriega et al., 2000, Khiroug et al., 2003, Karadsheh et al., 2004, Fayuk and Yakel, 2005, 2007, Jia et al., 2010), this effect is most probably accomplished by calcium concentration alterations.

At first glance, the impact of nicotinic receptor enhancement on plasticity in the present experiment is not completely compatible with the direction of effects obtained in the above-mentioned animal experiments, especially with regard to LTD-induction. However, the key for understanding the results might be the non-linear impact of calcium on plasticity. Whereas low intraneuronal calcium enhancement induces LTD, high concentrations induce LTP. In between, a "no man´s land" does exist, in which no plasticity results, and very high calcium concentrations might also prevent plasticity because of activation of hyperpolarizing potassium channels (Lisman, 2001, Misonou et al., 2004). Therefore, whereas both strains of experiments stress the role of nicotine receptors for plasticity, the reason for differently directed results of animal and human experiments might be different amounts of calcium influx caused by the respective receptor agonists, and plasticity induction procedures.

The reason that low dosage varenicline, which are 10 times lower than the single oral dosage (1mg) administered in smokers to support cessation of tobacco consumption (Faessel et al., 2010), had no significant effect on plasticity is most probably that this dosage did not suffice to activate nicotinic receptors to an amount at which these induce relevant intraneuronal calcium concentration alterations. The plasticity-abolishing effects of the medium and high dosages of the drug with regard to excitability-diminishing plasticity, and tDCS-induced facilitatory plasticity go in line with the results of previous studies (Grundey et al., 2012b, Thirugnanasambandam et al., 2012), where global nicotinic receptor activation resulted in abolishment of these kinds of plasticity. Therefore, it is plausible that at least a part of the impact of nicotine on plasticity is driven by $\alpha_4\beta_2$ and α_7 receptors. As varenicline is a full agonist of α_7 and potent partial agonist of $\alpha_4\beta_2$ receptors, with a far greater affinity (4000-5000 fold) to $\alpha_4\beta_2$ as compared to α_7 receptors (Avalos et al., 2002, Jensen et al., 2005, Mihalak et al., 2006, Rollema et al., 2010), the $\alpha_4\beta_2$ receptor might have a larger relevance for the results. Due to the above-mentioned calcium hypothesis, the most probable explanation for the abolishment of LTD-like plasticity by the medium dosage of the drug is that here nicotinic receptor activation drove calcium concentrations in the respective "no man´s lands". For the abolishment of the non-focal LTP-like plasticity induced by anodal tDCS under high-dosage varenicline, the same mechanism might apply. In contrast, the PAS25-induced excitability enhancement was not affected by any dose of varenicline. This can be explained by differences between the stimulation-inducing protocols.

Neuroplastic changes via tDCS are achieved by long-lasting, tonic depolarization of large population of neurons and activation of voltage-dependent Ca^{2+}-channels, whereas PAS only affects small group of neurons and causes short-lasting depolarizations. Therefore the amount of intracellular calcium increase may be smaller with regard to PAS as compared to tDCS and not sufficient to induce significant changes in neuroplasticity (Thirugnanasambandam et al., 2012).

This mechanism does however not explain the re-establishment of cathodal tDCS-induced LTD-like plasticity under the high dosage of the drug. Here it could be speculated that an antagonistic effect of varenicline on the respective nicotinic receptor, which takes place for higher dosages of the drug, resulted in reduced calcium influx, and thus a restitution of plasticity. These mechanistic explanations are however hypothetical at present, and should be explored more directly in future studies in humans, and animal models.

For the overall pattern of experimental results, varenicline applied in medium and high doses results in a focusing effect on facilitatory neuroplasticity, preserving focal, but abolishing non-focal facilitatory plasticity, similar to global nicotinic and cholinergic system activation (Kuo et al., 2007, Thirugnanasambandam et al., 2012). Such a focusing effect might be beneficial for task performance via enhancing the signal-to-noise ratio and can explain the positive nicotinic effect on cognitive functions (attention, working and episodic memory), where a stable processing of information is essential (Provost and Woodward, 1991, Kumari et al., 2003, Jubelt et al., 2008, Froeliger et al., 2009, Heishman et al., 2010, Mocking et al., 2012). Further behavioral experiments should be designed to explore this hypothesis.

General remarks

This study demonstrates that varenicline has a prominent impact on neuroplasticity in non-smoking humans, which is similar to that of nicotine application. Besides being an effective smoking cessation agent, varenicline is also suggested to have therapeutic effect in patients suffering from Alzheimer’s Disease (Kem, 2000, Jensen et al., 2005, Mihalak et al., 2006) and patients with schizophrenia during smoking abstinence (Hong et al., 2011, Liu et al., 2011, Shim et al., 2012). From this perspective, exploring the role of specific receptors ($\alpha_4\beta_2$ and possibly α_7

too) in the nicotinic effect on cognition and neuroplasticity is important and should be further addressed in future studies.

It has to be taken into account that the results of this study show only the impact of a single dose of varenicline on neuroplasticity. Many studies have shown that chronic exposure to nicotine can cause upregulation (Wonnacott, 1990, Buisson and Bertrand, 2001) and desensitization (Hsu et al., 1996, Fenster et al., 1997, Fenster et al., 1999) of nAChRs, therefore the effect of varenicline after chronic administration on neuroplasticity might be qualitatively different from that after an acute dose. This important aspect of nicotinic impact on neuroplasticity should also be explored in future studies.

Recent studies have shown that neuroplasticity, as well as verbal and working memory functions are reduced in smoking individuals after nicotine withdrawal and restituted by nicotine re-administration (Cole et al., 2010, Grundey et al., 2012a). Varenicline has also shown to improve working memory in nicotine abstinence (Patterson et al., 2009, Loughead et al., 2010). It might be interesting to explore if varenicline has similar restituting effects on plasticity, as shown for cognitive processes, in these individuals.

Limiting Conditions

A possible limitation to our study is that varenicline is an agonist with moderate affinity to 5-HT3 serotonin receptors (Lummis et al., 2011). 5-HT3 receptors have a facilitatory impact on plasticity (Normann et al., 2007, Nitsche et al., 2009, Batsikadze et al., In Press). However, concentrations of therapeutic unbound varenicline in the brain are insufficient for activation of these receptors (Rollema et al., 2011). Moreover, in a recently conducted study, the serotonin reuptake inhibitor citalopram enhanced tDCS-induced LTP-like plasticity, and converted LTD-like plasticity into facilitation (Nitsche et al. 2009). These results are qualitatively different to those obtained in the present study. Varenicline has also an impact on D2/3 dopamine receptor binding and availability in rats (Crunelle et al., 2009, Crunelle et al., 2011, Crunelle et al., 2012) and GABAergic synaptic transmission (DuBois et al., 2013), which have a major impact on stimulation-induced plasticity. It should be noted that also for these transmitters and receptors,

pharmacological modulations resulted in effects which clearly differ from those obtained under varenicline (Nitsche et al., 2004, Kuo et al., 2008, Monte-Silva et al., 2009, Monte-Silva et al., 2010). Nevertheless, in order to explore the complex interplay of neuromodulatory systems on nicotine-modulated plasticity, future studies should use approaches combining pharmacological interventions with neuroimaging.

Another limitation is that the specific neurophysiological mechanisms underlying the nicotinic impact on various corticospinal and intracortical excitability parameters were not investigated. We did not perform these measures in the present study, because this would have made it impossible to explore the detailed time-course of plasticity. However, it would be important to explore the effect of varenicline on cholinergic activity e.g. by monitoring short-latency afferent inhibition (SAI) and on GABAergic and glutamatergic transmission by measuring short-latency intracortical inhibition (SICI) and intracortical facilitation (ICF) (Ziemann et al., 1996, Di Lazzaro et al., 2002, Di Lazzaro et al., 2005, Paulus et al., 2008), to unravel the physiological background of the respective effects.

Disclosure

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Table 1. MEP amplitudes and stimulation intensity before and after varenicline administration.

Stimulation	TMS Parameter	Medication condition	Baseline 1	Baseline 2	Baseline 3
Cathodal tDCS	MEP	0.1mg	0.95 ± 0.07	0.95 ± 0.07	0.96 ± 0.13
		0.3mg	1.00 ± 0.11	0.94 ± 0.17	0.96 ± 0.14
		1.0mg	0.92 ± 0.06	0.98 ± 0.12	0.98 ± 0.11
		Placebo	0.95 ± 0.07	0.94 ± 0.09	0.95 ± 0.08
	%MSO	0.1mg	53.67 ± 6.76	53.67 ± 6.76	53.67 ± 6.76
		0.3mg	53.58 ± 7.25	53.58 ± 7.25	53.92 ± 7.63
		1.0mg	54.00 ± 7.39	54.00 ± 7.39	54.00 ± 7.22
		Placebo	53.50 ± 7.18	53.50 ± 7.18	53.58 ± 7.24
Anodal tDCS	MEP	0.1mg	0.99 ± 0.13	0.92 ± 0.17	0.95 ± 0.13
		0.3mg	0.98 ± 0.10	1.00 ± 0.25	0.95 ± 0.07
		1.0mg	0.98 ± 0.09	1.01 ± 0.42	0.98 ± 0.09
		Placebo	0.92 ± 0.08	0.97 ± 0.12	0.98 ± 0.11
	%MSO	0.1mg	53.67 ± 6.81	53.67 ± 6.81	53.83 ± 6.79
		0.3mg	52.83 ± 6.81	52.83 ± 6.81	52.67 ± 6.85
		1.0mg	53.33 ± 7.48	53.33 ± 7.48	53.42 ± 7.51
		Placebo	53.25 ± 7.90	53.25 ± 7.90	53.33 ± 7.91
PAS10	MEP	0.1mg	0.96 ± 0.12	0.95 ± 0.29	1.01 ± 0.10
		0.3mg	1.03 ± 0.10	0.99 ± 0.18	0.96 ± 0.11
		1.0mg	1.00 ± 0.12	1.00 ± 0.16	1.00 ± 0.08
		Placebo	0.97 ± 0.08	0.99 ± 0.07	0.99 ± 0.07
	%MSO	0.1mg	51.00 ± 9.05	51.00 ± 9.05	51.33 ± 9.82
		0.3mg	52.17 ± 9.33	52.17 ± 9.33	52.25 ± 9.29
		1.0mg	50.83 ± 9.46	50.83 ± 9.46	50.83 ± 9.77
		Placebo	51.83 ± 9.23	51.83 ± 9.23	51.83 ± 9.23
PAS25	MEP	0.1mg	0.99 ± 0.12	0.99 ± 0.14	1.00 ± 0.11
		0.3mg	1.00 ± 0.09	1.00 ± 0.17	1.00 ± 0.12
		1.0mg	0.99 ± 0.10	1.04 ± 0.16	1.02 ± 0.10
		Placebo	0.99 ± 0.10	0.98 ± 0.11	0.98 ± 0.11
	%MSO	0.1mg	52.83 ± 8.28	52.83 ± 8.28	53.08 ± 8.55
		0.3mg	52.00 ± 9.72	52.00 ± 9.72	51.92 ± 9.69
		1.0mg	52.75 ± 9.12	52.75 ± 9.12	52.75 ± 9.08
		Placebo	52.58 ± 8.56	52.58 ± 8.56	52.58 ± 8.56

Shown are the mean MEP amplitudes ± S.D. and stimulation intensity (percentage of maximum stimulator output, %MSO) mean ± S.D. of baselines 1, 2 and 3. The intensity of TMS was adjusted to elicit MEPs with peak-to-peak amplitude of ~1mV (baseline 1). A second baseline (baseline 2) was recorded three hours after varenicline or placebo intake to determine the impact of the drug on cortical excitability and adjusted if necessary (baseline 3). Student´s t-tests revealed no significant differences between conditions ($p > 0.05$).

Table 2. Results of the repeated measures ANOVA.

Experiment	Factor	Df	F	p
tDCS	Medication	3	0.596	0.622
	Stimulation	1	117.900	**<0.001***
	Time	14	1.233	0.257
	Medication x Stimulation	3	5.050	**0.005***
	Medication x Time	42	0.931	0.598
	Stimulation x Time	14	10.013	**<0.001***
	Medication x Stimulation x Time	42	2.375	**<0.001***
PAS	Medication	3	0.838	0.488
	Stimulation	1	19.134	**0.003***
	Time	14	1.285	0.230
	Medication x Stimulation	3	1.468	0.252
	Medication x Time	42	0.871	0.699
	Stimulation x Time	14	19.064	**<0.001***
	Medication x Stimulation x Time	42	1.476	**0.035***

*Significant results at $p < 0.05$.

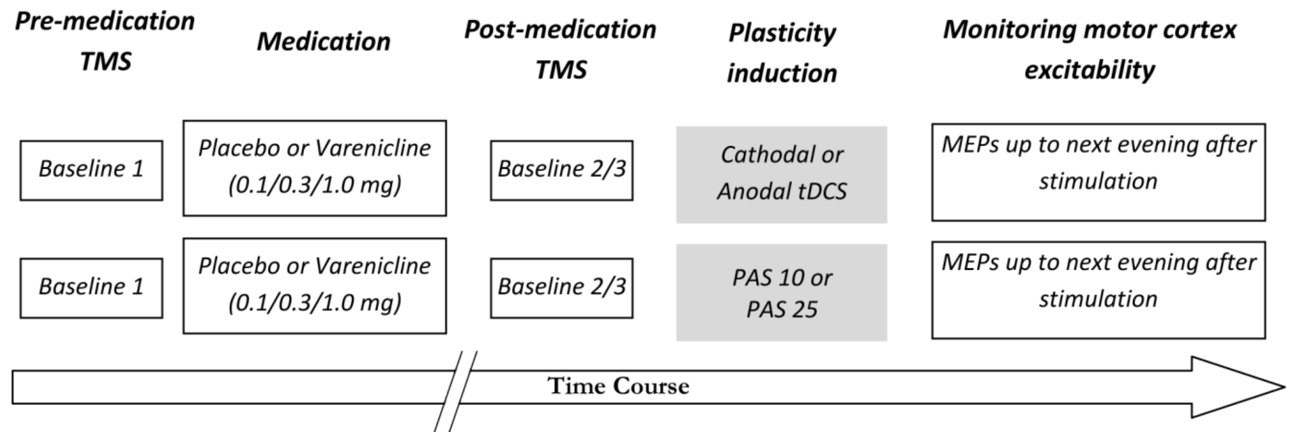
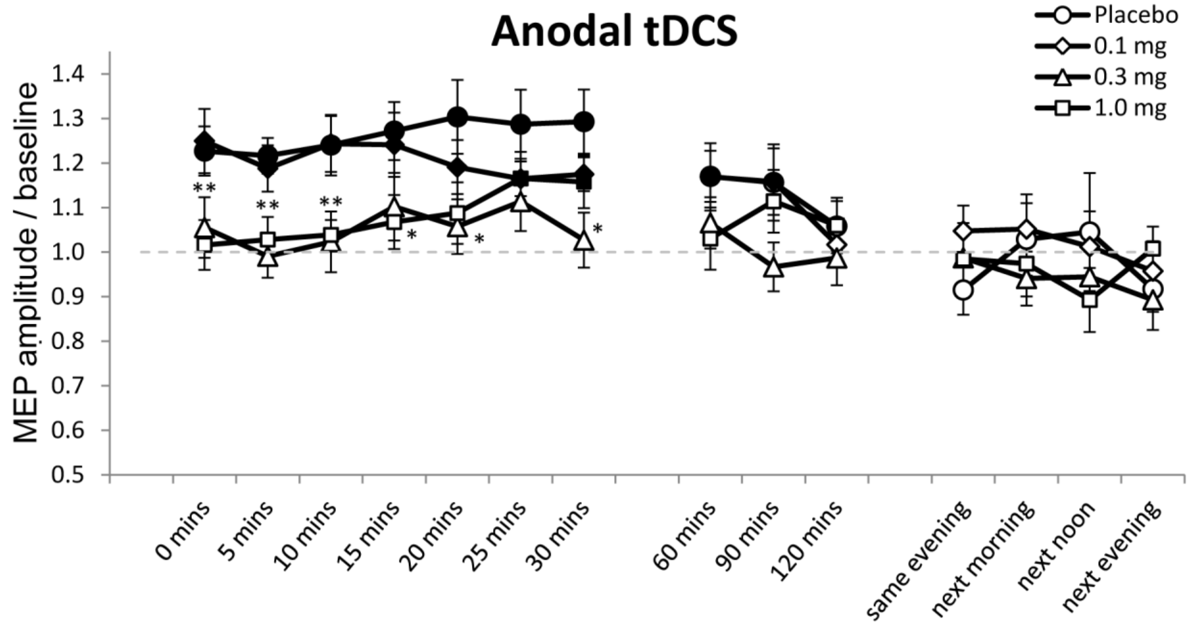


Figure 1. Course of the study. In the beginning of each session, before administration of varenicline or placebo medication, 25 baseline single pulse MEPs were recorded at an intensity to elicit MEPs with peak-to-peak amplitudes of on average 1 mV. Three hours later, a second baseline was recorded to explore the effect of medication on cortical excitability, and adjusted, if necessary (third baseline). Afterwards, tDCS (cathodal or anodal) or PAS (PAS10 or PAS25) was administered and 25 MEPs were recorded immediately after stimulation and at time points of 5, 10, 15, 20, 25, 30, 60, 90 and 120 minutes after plasticity induction. Further TMS measurements were conducted in the evening of the same day (SE), next morning, at ~9:00AM (NM), next noon, at ~12:00PM (NN) and next evening, at ~6:00PM (NE).

A)



B)

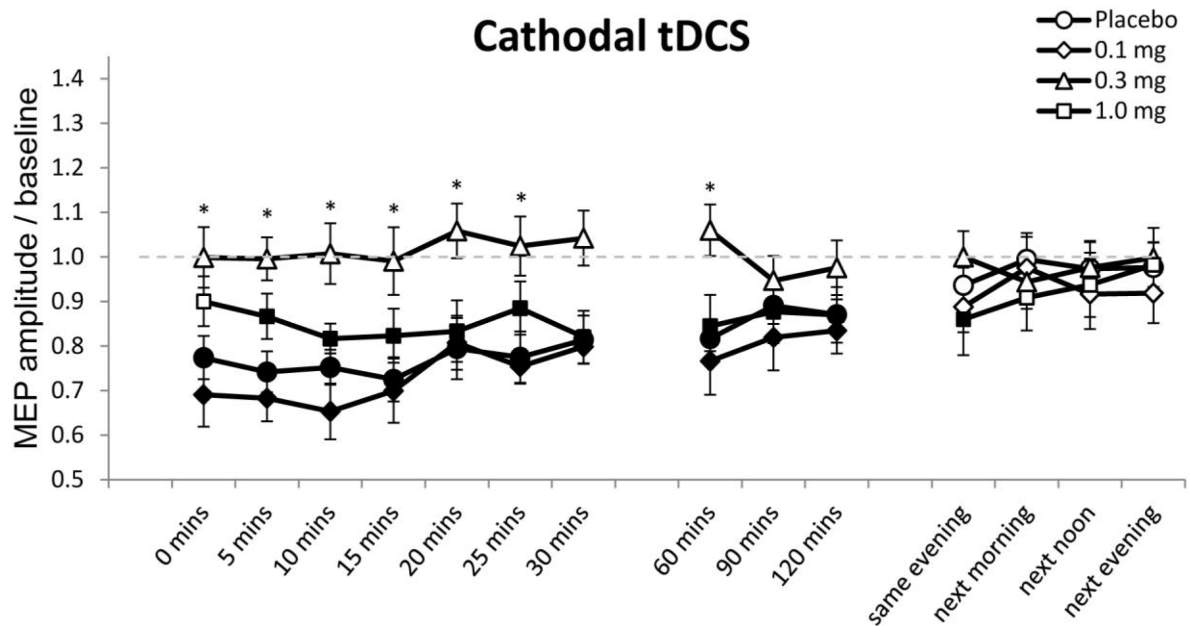
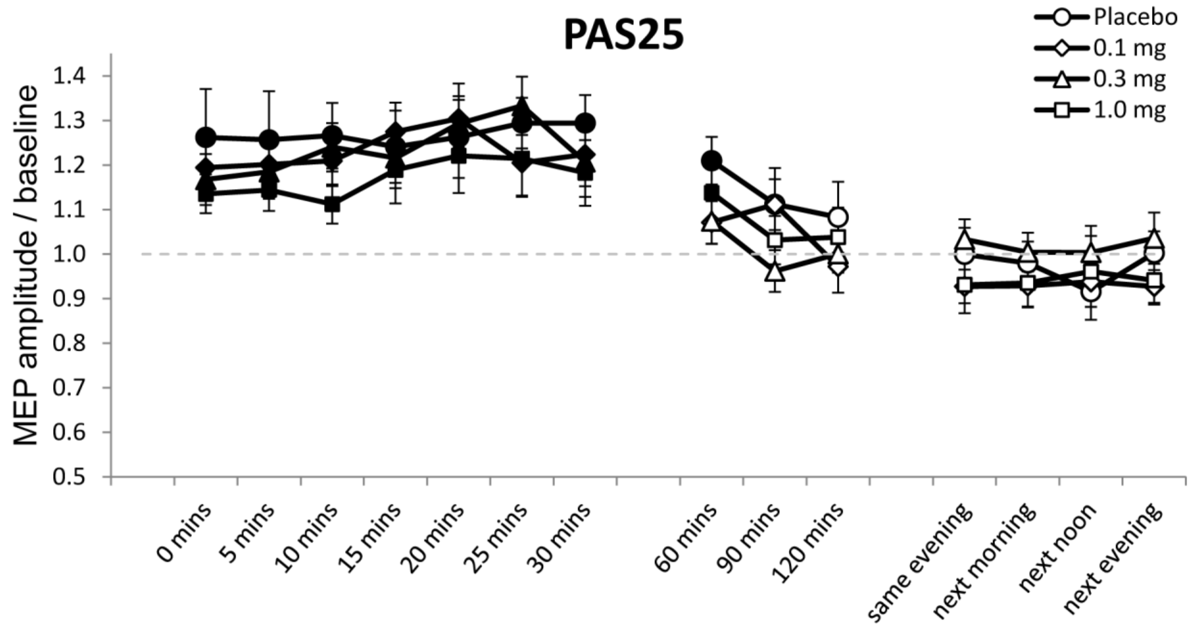


Figure 2. Impact of varenicline on tDCS-induced neuroplasticity. Shown are baseline-normalized MEP amplitudes after plasticity induction by anodal (**A**) and cathodal (**B**) tDCS under 0.1mg,

0.3mg, 1.0mg varenicline or placebo medication conditions up to the evening of the post-stimulation day. **A.** In the placebo and 0.1mg varenicline medication conditions, anodal tDCS induced a significant excitability elevation up to 60 minutes after stimulation, which was abolished by 0.3mg and 1.0mg varenicline. **B.** In the placebo, 0.1mg and 1.0mg varenicline medication conditions, cortical excitability was significantly reduced after cathodal tDCS administration. This effect was abolished by 0.3mg varenicline. Error bars indicate S.E.M. Filled symbols indicate significant differences of post-stimulation MEP amplitudes from respective baseline values; asterisks indicate significant differences between the drug and placebo medication conditions at the same time points (Student's t-test, two tailed, paired samples, $p < 0.05$).

A)



B)

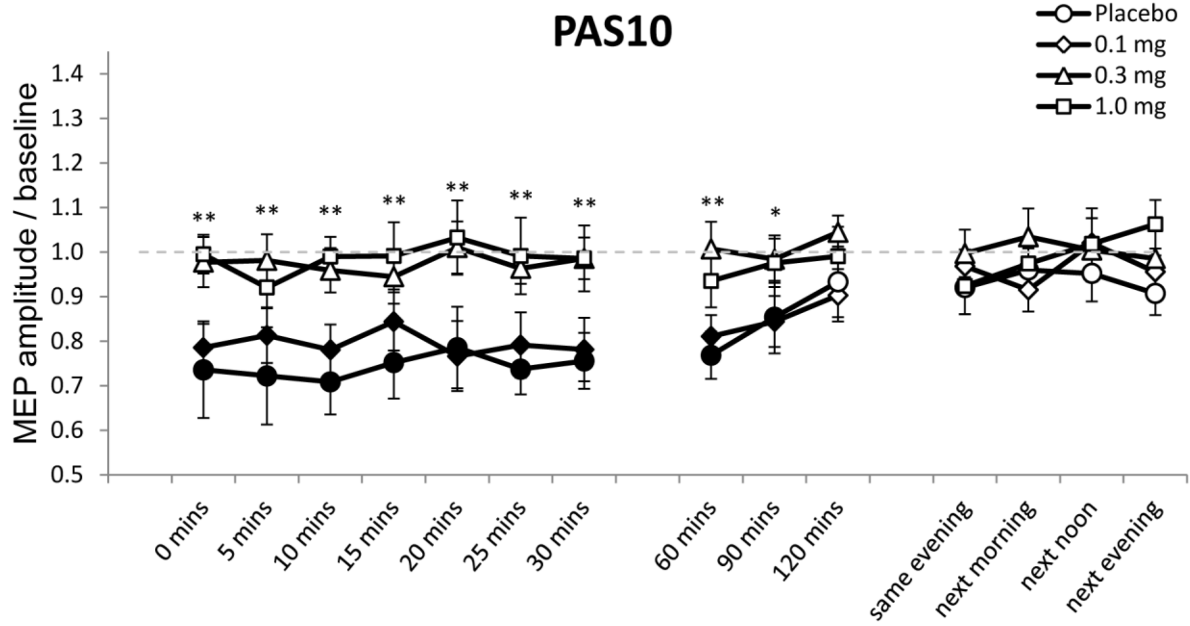


Figure 3. Impact of varenicline on PAS-induced neuroplasticity. Shown are baseline-normalized MEP amplitudes after plasticity induction by PAS25 (**A**) and PAS10 (**B**) under 0.1mg, 0.3mg,

1.0mg varenicline or placebo medication conditions up to the evening of the post-stimulation day. **A.** Cortical excitability was significantly elevated up to 30 minutes in all medication conditions after PAS25 administration. **B.** In the placebo and 0.1mg varenicline medication conditions, cortical excitability was significantly reduced up to 60 minutes after PAS10. 0.3mg and 1.0 mg varenicline abolished the above mentioned excitability diminution. Error bars indicate S.E.M. Filled symbols indicate significant differences of post-stimulation MEP amplitudes from respective baseline values; asterisks indicate significant differences between the drug and placebo medication conditions at the same time points (Student's t-test, two tailed, paired samples, $p < 0.05$).

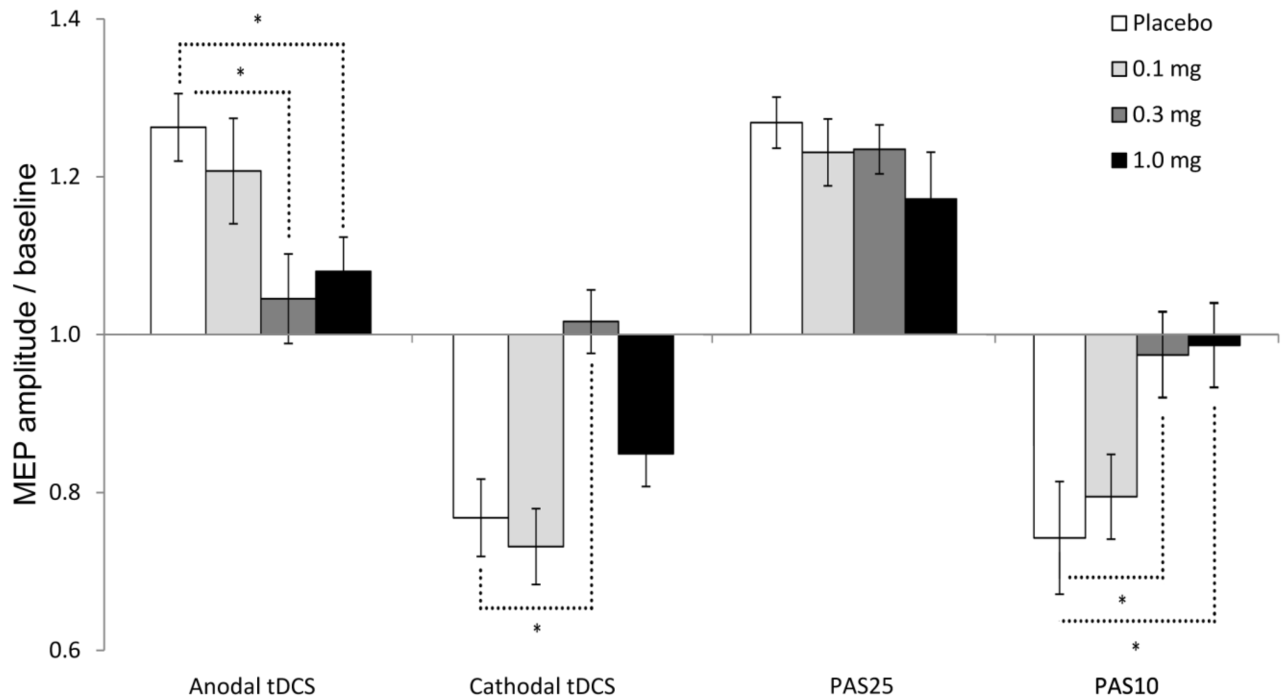


Figure 4. Both, anodal and cathodal tDCS-induced plasticity is abolished by 0.3mg varenicline, Anodal tDCS-induced excitatory plasticity is abolished and cathodal tDCS-induced inhibitory plasticity is preserved by 1.0mg varenicline. 0.1mg varenicline has no impact on stimulation-induced plasticity. Medium and high doses of varenicline abolished PAS10-induced inhibitory plasticity. Varenicline at any doses did not have an impact on PAS25 induced excitability enhancement. Each column represents the mean of baseline-normalized MEP ± S.E.M. amplitudes until 30 minutes after stimulation; Asterisks indicate significant differences between drug and placebo conditions (Student's t-test, two tailed, paired samples, $p < 0.05$).

Chapter 3 – Summary and Conclusions

The studies presented in this thesis explore different aspects of neuroplasticity in the human brain. The first study demonstrated a non-linear association between tDCS intensity and its after effects. An enhancement of tDCS intensity is not always accompanied by an increase of efficacy of stimulation, but might also shift the direction of excitability alterations as in case of 2mA cathodal stimulation. Similar non-linear associations between stimulation intensity and after-effects have been previously shown for other non-invasive brain stimulation protocols (rTMS, tRNS, tACS) (Doeltgen and Ridding, 2010, Moliadze et al., 2012). This finding should especially be considered with regard to clinical application of the stimulation technique. These results also imply that before therapeutical administration of modified stimulation protocols, it is necessary to study their physiological effects. The results of this study also lead to the assumption that in healthy individuals a “ceiling effect” exists that cannot be surpassed by simply increasing the intensity and/or duration of the stimulation. For achieving desired longer and stronger stimulation after-effects, the use of repeated stimulation protocols and pharmacological interventions is suggested (Nitsche et al., 2004, Kuo et al., 2008, Nitsche et al., 2009, Monte-Silva et al., 2010a, Monte-Silva et al., 2013). These non-linear physiological effects are reflected by the results of cognitive studies, where the impact of different tDCS intensities is even less uniform. Some studies show performance improvement via 2mA tDCS compared to 1mA (Iyer et al., 2005, Moos et al., 2012), the opposite effect (Hoy et al., 2013) or no difference (Teo et al., 2011). Beyond non-linear effects of tDCS applied with different intensities on the affected neurons, and an interaction between stimulation- and cognition-dependent neuronal activation, which might differ from the effect of “tDCS-only” conditions, another reason for such non-linear effects might be that increased intensity of the transcranially injected electric current could lead to increased electric field strength in subcortical regions and additional recruitment of adjacent, non-target brain regions, resulting in altered plasticity and functional connectivity (Boros et al., 2008, Datta et al., 2009, Polania et al., 2011a, Polania et al., 2011b, Polania et al., 2012, Kessler et al., 2013). However, these hypotheses are speculative and should be subject of future experiments.

In contrast to these results, several clinical studies (Boggio et al., 2006, Fregni et al., 2006a, Fregni et al., 2006b, Brunoni et al., 2011, Brunelin et al., 2012) demonstrate a positive impact of 2mA stimulation. In neuropsychiatric diseases, pathologically altered brain plasticity, and

activity, and thus an altered pre-stimulation state of brain physiology, could be the reason for the effectiveness of stronger/longer tDCS protocols, broadening the range in which plasticity alterations aimed for can be accomplished. Previous studies showed clearly that the basal state of cortical activity, and excitability have a relevant impact on the kind of plasticity induced (Siebner et al., 2004, Fricke et al., 2011). Thus the results of our study, conducted in healthy young participants and using the primary motor cortex as a model, might not translate one-to-one to patient populations or cognitive experiments.

The second and third studies addressed the knowledge gaps with regard to the involvement of certain neuromodulatory systems and receptors in specific plasticity types. In principal accordance to previous studies (Normann et al., 2007, Nitsche et al., 2009), the results of the second study demonstrate an enhancement of LTP-like plasticity and a reduction of LTD-like plasticity by serotonin, therefore shifting resulting net plasticity into the direction of facilitation. This might explain the positive effect of serotonin enhancers on rehabilitation in diseases, such as stroke and depression, accompanied by enhanced inhibitory and reduced excitatory plasticity (Foy et al., 1987, Dam et al., 1996, Traversa et al., 1997, Xu et al., 1997, Traversa et al., 1998, Pariente et al., 2001, Schaechter, 2004, Chollet et al., 2011, Player et al., 2013). Given that PAS-induced plasticity is related to STDP (Stefan et al., 2000, Wolters et al., 2003), these results are also helpful for explaining the positive serotoninergic impact on cognitive processes (Loubinoux et al., 1999, Loubinoux et al., 2002a, Loubinoux et al., 2002b, Loubinoux et al., 2005).

It is hypothesized that 5-HT2 and 5-HT3 are candidate receptors in serotoninergic modulation of plasticity, as they modulate intracellular Ca^{2+} concentration (Reiser et al., 1989, Ronde and Nichols, 1998). However, their specific impact on plasticity is not yet clear and has to be studied in future experiments using respective agonist and antagonist pharmacological agents and different plasticity-inducing protocols.

The third study sheds some light on the dose-dependency of $\alpha_4\beta_2$ nicotinic receptor activation on neuroplasticity. So far, the involvement of specific receptors in nicotine-modulated human brain plasticity remained unclear. In this project, we used different doses of the $\alpha_4\beta_2$ nicotinic receptor partial agonist varenicline (Mihalak et al., 2006) and studied their impact on stimulation-induced non-focal and focal plasticity of the human primary motor cortex. The

results of this study show that a low dose of varenicline has no impact on cortical plasticity, while a medium dose preserves only focal facilitatory plasticity, whereas it abolishes other plasticity types. Varenicline in high doses preserved focal facilitatory and non-focal inhibitory plasticity. The results obtained under the medium-dose are identical to those of global nicotinic activation (Thirugnanasambandam et al., 2012). In high doses, preservation of non-focal inhibitory plasticity by varenicline could be explained by its antagonist effect in high dosages and therefore reduced calcium influx. The results of this study are in accordance with a crucial importance of this receptor for the modulatory impact of nicotine on plasticity, which most probably is driven by intracellular calcium alterations. Besides $\alpha_4\beta_2$, α_7 nAChRs also have Ca^{2+} channel properties (Burnashev, 1998, Dajas-Bailador and Wonnacott, 2004), therefore it is essential to study their impact on neuroplasticity in order to fully explore the contribution of nicotinic receptors (Matsuyama and Matsumoto, 2003).

With regard to the functional importance of this finding, it is relevant to notice that nicotine withdrawal impairs memory functions and neuroplasticity in smoking individuals and its readministration restitutes them (Cole et al., 2010, Grundey et al., 2012). Taking into account that varenicline is a popular smoking cessation drug (Coe et al., 2005) and has been shown to improve memory functions in nicotine abstinence (Patterson et al., 2009, Loughead et al., 2010), its possible restitutive effect on tobacco consumption-related impaired plasticity and cognitive functions might contribute to diminishing the probability to relapse in smoking addicts after cessation, which will be interesting to explore in future experiments.

Possible limitations of the second and third studies is that we administered single oral doses of citalopram and varenicline. Many studies demonstrate that chronic exposure to neuromodulatory substances can lead to desensitization or up- and downregulation of receptors (Wonnacott, 1990, Blier and Bouchard, 1994, Pineyro et al., 1994, Hsu et al., 1996, Fenster et al., 1997, Fenster et al., 1999, Buisson and Bertrand, 2001, Yamane et al., 2001), therefore the effects of these substances on neuroplasticity under chronic administration, as relevant for clinical application, could be qualitatively different from those after a single-dose. This important aspect should also be explored in future studies.

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