New non-invasive transcranial stimulation techniques in neuroplasticity research

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Original papers listed in the thesis

I. Antal A, Boros K, Poreisz C, Chaieb L, **Terney D**, Paulus W. Comparatively weak after-effects of transcranial alternating current stimulation (tACS) on cortical excitability in humans. *Brain Stimulation. 2008 Apr; 1(2):97-105.*

II. **Terney D,** Chaieb L, Moliadze V, Antal A, Paulus W. Increasing human brain excitability by transcranial high-frequency random noise stimulation. *The Journal of Neuroscience. 2008 Dec; 28(52):14147-14155.*

Other publications related to the dissertation

Antal A, **Terney D**, Poreisz C, Paulus W. Towards unravelling task-related modulations of neuroplastic changes induced in the human motor cortex. *European Journal of Neuroscience. 2007; 26:2687-2691.*

Terney D, Bergmann I, Poreisz C, Chaieb L, Boros K, Nitsche MA, Paulus W, Antal A. Pergolide increases the efficacy of cathodal direct current stimulation to reduce the amplitude of laser-evoked potentials in humans. *Journal of Pain and Symptom Management. 2008; 36(1):79-91.*

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Figure 8. TRNS of the primary motor cortex improves implicit motor learning in its early phase. Reaction times decrease faster in the tRNS condition when compared to the sham stimulation condition (upper figure). Moreover, the RT difference comparing block 5, and 6, which indicates implicit sequence learning, is bigger for the tRNS condition, when compared to sham condition. The asterisk indicates a significant difference regarding reaction time differences between blocks 5 and 6, and between RN and sham stimulation. In one and two hours post-stimulation this significant difference was no longer detectable (lower figures).

List of abbreviations

Ach: acetylcholine
AE: after-effect
AMT: active motor threshold
BCM: Bienenstock-Cooper-Munro
BOLD: blood oxygenation level dependent
CSP: cortical silent period
DA: dopamine
D1-receptor: dopamine-receptor type 1
D2-receptor: dopamine-receptor type 2
DC: direct current
ECBs: endogenous cannabinoids
EEG: electroencephalography
EMG: electromyogram
ER: error rate
FDI: first dorsal interosseus
FFT: Fast Fourier Transformation
ICF: intracortical facilitation
ISI: interstimulus interval
iTBS: intermittent theta burst stimulation
LICI: long-interval intracortical inhibition
LTD: long-term depression
LTP: long-term potentiation
M1: primary motor cortex
MEP: motor evoked potential
NMDA: N-methyl-D-aspartate
NSE: neuron-specific enolase
RMT: resting motor threshold
RT: reaction time
rTMS: repetitive transcranial magnetic stimulation
SEM: measurement of standard error
SI1mV: 1mV peak-to-peak amplitude
SICI: short-interval intracortical inhibition
SRTT: serial reaction time task
tACS: transcranial alternating current stimulation
TBS: theta burst stimulation
tDCS: transcranial direct current stimulation
TMS: transcranial magnetic stimulation
tRNS: transcranial random noise stimulation
tSDCS: transcranial sinusoidal direct current stimulation

Összefoglalás

Az elmúlt 20 évben számos nem-invazív transzkraniális stimulációs technika került bevezetésre az idegtudományok területét érintő alap- és klinikai kutatásban. A legismertebb neuroplaszticitás indukálása és fokozása céljából használt eszköz ezek közül a repetitív transzkraniális mágneses stimuláció (rTMS), valamint a transzkraniális egyenáram-ingerlés (tDCS).

Vizsgálatsorozatunk során új elektromos stimulációs technikákat teszteltünk, transzkraniális váltóáram stimulációt (tACS), valamint transzkraniális random zaj ingerlést (tRNS) vizsgáltunk elektrofiziológiai- és pszichofiziológiai módszerek segítségével. Kísérleteink első csoportjában 48 egészséges alany bevonásával a tDCS spektrumát terjesztettük a tACS felé. 10 Hz elsődleges motoros kérgen (M1) történő ingerlés a motoros kiváltott válaszok (MEP) amplitudóját csökkentette, emellett gyorsabb implicit motoros tanulást eredményezett a pszichofiziológiai tesztek használata során. Vizsgálataink egy részében a tACS-t anódális és katódális DC stimulációval kombináltuk. Ezekben a vizsgálatokban a MEP-ek amplitudója anódális 10- és 15 Hz-es ingerlést követően emelkedett.

Kísérleteink második csoportja 80 egészséges önkéntesen a tRNS technika utóhatásait vizsgálta. A tRNS a kortikális excitabilitás fokozódását eredményezte, mely emelkedés a stimulációt követően 60 percig szignifikáns mértékű volt. Az észlelt excitabilitás fokozódás mind az elektrofiziológiai-, mind pszichofiziológiai feladatok végzése során észlelhető volt. A kortikális ingerlékenység fokozódásáért eredményeink alapján elsősorban a magasabb frekvenciatartomány (100-640 Hz) tehető felelőssé.

Összegezve, a tACS és a tRNS hasznos eszközként szolgálhat neurofiziológiai alapkísérletek és klinikai kutatások során. Eredményeink alapján úgy tűnik, hogy a tRNS potenciális terápiás hatása az rTMS és tDCS terápiás hatásával mérhető. További vizsgálatok végzése azonban elengedhetetlen a biztonságos alkalmazási tartomány, illtve a potenciális klinikai használhatóság megállapítása végett.

Summary

For more than 20 years, non-invasive transcranial stimulation techniques like repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS) have been used to induce and potentiate neuroplastic-like effects in the human cortex, leading to synaptic alterations, namely the experience- and activity-dependent modification of synaptic transmission.

In our experiments we introduce novel methods of electrical stimulation, namely transcranial alternating current stimulation (tACS) and transcranial random noise stimulation (tRNS). In the first group of our experiments we extended the tDCS technique to tACS. A marked decrease in motor evoked potential (MEP) amplitudes of about 20%, and improved implicit motor learning was observed after 10 Hz AC stimulation over the primary motor cortex (M1) in altogether 48 healthy subjects. If anodal or cathodal DC stimulation was superimposed on 5, 10 and 15 Hz AC stimulation, the MEP amplitudes were increased after anodal 10 and 15 Hz stimulation.

In the second group of studies, we introduce tRNS, whereby an alternating current with a random electrical oscillation-spectrum is applied over the M1. TRNS induced consistent excitability increases last 60 minutes post-stimulation. These effects have been observed in 80 subjects through both physiological measures (MEPs) and behavioural tasks (SRTT). Higher frequencies (100-640 Hz) appear to be responsible for generating this excitability increase.

Our results suggested that transcranial application of weak AC and RN currents may appear to be a tool for basic and clinical research in diseases with altered EEG activity. TRNS appears to possess at least the same therapeutic potential as rTMS or tDCS, while furthermore avoiding the constraint of current flow direction sensitivity characteristic of tDCS. Further studies are required to extend cautiously the safety range and uncover its influence on neuronal circuitries.

Introduction

Neuroplasticity is an ongoing, self-organizing, adaptive process widespread in cortical areas; it allows the brain to learn and adapt to new environmental situations. External influences on neuroplastic processes may be used for the functional improvement of diseases, in particular for improving cortical functions such as learning. Several methods exist to influence excitability of the brain by external or transcranial stimulation. The most well-known method to influence excitability of the brain by external means is transcranial magnetic stimulation (TMS).

Another approach, weak transcranial direct current stimulation (tDCS) of the brain was investigated intermittently within the last four decades, but entered into neurobiological and clinical plasticity research only after its efficacy for modulating neuroplasticity could be unambiguously quantified by comparing TMS induced MEPs before and after tDCS (Nitsche and Paulus, 2000, 2001).

Neuroplasticity in the central nervous system

Neuroplasticity is the ability of the nervous system to alter its functional organization as a result of experience (Nudo, 2006). It can be a part of either normal learning procedures or recovery after injuries. Such injuries can occur following stroke, hypoxic events, or trauma (Hallett, 2001; Siebner et al., 2004; Karmarker and Dan, 2006). Cortical plasticity is based on both cellular modifications and changes in neuronal networks (Karmarker and Dan, 2006).
Several types of so-called 'injury-induced plasticity', or rearrangement of the nervous system in response to injury, have been known for decades to generate functional recovery. Among these mechanisms are the 'unmasking' of synapses or pathways that may ordinarily be inactive; 'denervation hypersensitivity', in which the target of a partially lesioned projection produces a great number of receptors to bind to a reduced number of available neurotransmitter molecules; and 'compensatory collateral sprouting', wherein the injured distal components of axons that are spared by a lesion sprout to occupy adjacent synapses vacated by a lesioned neighbouring axon (Hámori et al., 1990; Hallett, 2001).

The cellular mechanisms of short-term neuroplastic changes are based on different mechanisms (Hallett, 2001), for example, unmasking. The unmasking form of plasticity can occur very rapidly -within minutes of an injury- and it is the change in the balance between

excitation and inhibition. A change in neuronal membrane excitability may occur via voltage-gated channels, and most likely via sodium channels. Long-term potentiation (LTP) and long-term depression (LTD) are the fast enhancement and diminution of already existing synapses. However, several studies have shown morphologic evidence for neuroplasticity, which requires a longer period of time (formation of new synapses and sprouting of new axon terminals). Hámori et al. (1990) demonstrated synaptic regeneration in the adult central nervous system following deafferentation: axonisation of dendrites leads to the formation of new dendro-dendritic synapses and a reduction in the size of the denervated nerve cells, leading to the relative increase in density of the surviving axon terminals. Detection of calcium accumulation in the dendritic spines is a well-described method to demonstrate synaptogenesis under electron microscopy (Toni et al., 1999). Peripheral denervation can also lead to the rearrangement of the cortical homunculus in different sensory modalities via axonal sprouting (Elliott et al., 1996).

The role of neurotransmitters is also an essential one with regard to neuroplastic changes (Kuo et al., 2007). Acetylcholine (Ach) and dopamine (DA) have neuromodulatory effects on cortical excitability and synaptic plasticity leading to LTP, whereas glutamatergic processes participate in LTD. Dopamine plays a role in LTD processes by activating D2-receptors and leads to the release of endogenous cannabinoids (ECBs), inducing LTD in the striatum (Calabresi et al., 2007). ECBs participate in LTP also, as demonstrated in memory and learning procedures (Zhu, 2006). A recent study by Nitsche et al. (2009) showed a clear modulatory effect of the SSRI citalopram on tDCS-induced plasticity. Citalopram shifted plasticity in a facilitatory direction.

Transcranial stimulation techniques in humans

Transcranial magnetic stimulation

One aim of developing external stimulation methods in humans was to modify cerebral excitability in a non-invasive, painless, reversible, and selective way. The most well-known method used to influence excitability of the brain by external means is transcranial magnetic stimulation (TMS) introduced about 25 years ago, first in a single pulse mode (Barker et al., 1985). Single pulse TMS is widely used in the routine diagnosis of pathological changes of the corticospinal tract (e.g. amyotrophic lateral sclerosis, multiple sclerosis, compressive myelopathies) and to estimate its integrity (Wagner et al., 2007).

It was followed by various repetitive stimulation paradigms. RTMS is able to induce

externally triggered alterations in the spiking pattern of neuronal populations, and interrupts or excites neuronal firing in a spatially and temporally restricted route (Wagner et al., 2007; Antal et al., 2008). The magnetic field is able to pass through tissues with high resistance (bone, fatty acid) without being changed. The selective and transient effect of rTMS over the M1 can be quantified by measuring the amplitude of elicited single pulse MEPs (Barker, 1985; Priori et al., 1998; Nitsche and Paulus 2000; Nitsche et al., 2002). TMS has good temporal resolution; however, it produces only a short after-effect (AE).

Recently another repetitive stimulation paradigm was introduced, namely theta burst stimulation (TBS; Huang et al., 2005). Although TBS increased the efficacy of rTMS by reducing stimulus intensity and the number of pulses required for achieving similar after-effects, its upper safety limits are still unclear due to the potential risk of rTMS inducing seizures (Wassermann, 1998).

Transcranial direct current stimulation

When compared to pulsed rTMS, tDCS represents the other end of the stimulation spectrum by delivering continuous electric current which leads to "brain polarization". TDCS is able to induce long-lasting changes in cortical excitability in a reversible, relatively selective, painless and safe manner. The basic neuronal mechanisms of tDCS were first described in the late 1950's and 1960's (Bindman et al., 1964; Purpura and McMurtry, 1965; Creutzfeldt et al., 1962). Primarily, it causes polarity-dependent shifts of the resting membrane potential and consequently changes the firing rates of neurons under the electrodes, neuronal projections and subsequent connected cortical areas (Bindman et al., 1964; Purpura and McMurtry, 1965; Lang et al., 2005). Generally, M1 excitability is enhanced by anodal and decreased by cathodal stimulation (Nitsche and Paulus, 2000). Although in humans the modulatory effect of tDCS had first been demonstrated in the motor system, it also influences visual, somatosensory, prefrontal functions and pain sensation as well (Nitsche et al., 2003a; Rogalewski et al., 2004; Antal et al., 2006, Terney et al., 2008). It allows for diagnostic and interventional applications (Nitsche and Paulus, 2000; Liebetanz et al., 2002; Webster et al., 2006; Fregni and Pascual-Leone, 2007). They also offer a potential therapeutic use in neurorehabilitation, chronic pain, focal epilepsy and neuropsychiatric disorders (Webster et al., 2006; Fregni et al., 2006; Liebetanz et al., 2006; Antal et al; 2008).

As tDCS modulates cortical excitability, it may also induce and modify neuroplastic changes. Human pharmacological studies were implemented in order to clarify the molecular and receptor mechanisms of tDCS. Table 1 gives a brief overview of the pharmacological

approaches to DC stimulation.

Drug	Effect	Short-term anodal	Short-term cathodal	Long-term anodal	Long-term cathodal
carbamazepine	voltage-dependent Na-channel-blocker	↓	∅	↓	∅
flunarazine	Ca^{++}-channel blocker	↓	∅	↓	∅
dextromethorphan	NMDA-receptor antagonist	∅	∅	↓	↓
d-cycloserine	NMDA agonist	↑	∅	↑	∅
lorazepam	GABA-A agonist	↑	∅	∅	∅
sulpiride	D2-receptor antagonist	∅	∅	↓	↓
pergolide	D1-receptor agonist	∅	↑	∅	↑
rivastigmine	ACh-estherase inhibitor	↓	↑	↓	↑
amphetamine	increases catecholamine	-	-	↑	∅
ropinirole	D2/D3 dopamine agonist	biphasic response: ↓: low and high dosages , ↑: medium dosage - prolonged inhibition after cathodal tDCS-			
citalopram	serotonin reuptake blocker	↑	↓	↑	↓

Table 1. This table represents the pharmacological approach concerning DC stimulation in long- and short-term anodal and cathodal stimulation.
-: not examined, ↑: the drug has increased the tDCS-induced effect, ↓: the drug has decreased the tDCS-induced effect, ∅: no effect.

Aim of the studies

The aim of our experiments was to introduce novel methods of non-invasive electrical stimulation. In our first study we expand further the stimulation spectrum between DC and AC stimulation. Transcranial alternating current stimulation (tACS) of the brain is a new technique. It intends to interfere with ongoing oscillations in the brain. These have mainly been discussed in context with the "binding hypothesis" (Singer, 2001). According to this hypothesis it is assumed that no single cell is able to reflect a single perception ("grandmother cell") or event.

Instead, different specialized brain areas have to be bound together by oscillations mainly in the gamma range. These fluctuating oscillations are suggested to provide a momentary functional network capable of solving any higher cognitive task required. External application of tACS could be able to interfere with these oscillations and might allow an experimental validation of the "binding hypothesis". This technique may also be important for neuropsychiatric disorders, as it has been concluded that measures of gamma synchrony offer a valuable window into the core integrative disturbance in schizophrenia (e.g. Lee et al., 2003). Recently it was shown that inducing slow oscillation-like potential fields by transcranial application of oscillating potentials (0.75 Hz) during early nocturnal non-rapid-eye-movement sleep, that is, a period of emerging slow wave sleep, enhances the retention of hippocampus-dependent declarative memories in healthy humans (Marshall et al., 2006). The slowly oscillating potential stimulation induced an immediate increase in slow wave sleep, endogenous cortical slow oscillations and slow spindle activity in the frontal cortex. Brain stimulation with oscillations at 5 Hz; another frequency band that normally predominates during rapid-eye-movement sleep, decreased slow oscillations and left declarative memory unchanged. Intracellular and EEG recordings in animals (Destexhe et al., 1999) have shown that modulation of the excitability of cortical pyramidal cells generates a powerful and coherent feedback to the thalamus, resulting in highly coherent oscillations similar to those measured during natural sleep. These experiments are compatible with a role for the cortex in triggering and synchronizing oscillations generated in the thalamus, through cortico–thalamo–cortical loops, thus providing a possible cellular mechanism to explain the genesis of large-scale coherent oscillations in the thalamocortical system. By stimulating the sensorimotor cortex using tACS, oscillations can be triggered and may also reset the ongoing rhythmic activity of a local pacemaker with a consequent synchronization of oscillations.

To investigate the aftereffects of tACS we assayed a frequency spectrum between 1 and 45 Hz using transcranial electrical stimulation and analysed MEPs and EEG-spectra before and after AC stimulation, with and without an anodal and cathodal DC shift. Furthermore, on a behavioural level we studied AC-driven changes in performance during a variant of the serial reaction time task (SRTT) (Nissen and Bullemer, 1987; Exner et al., 2002; Nitsche et al., 2003a), which is a standard paradigm to test implicit motor learning. In this task, subjects perform finger movements repetitively without being aware of a sequential order. We applied tACS or sham stimulation to the M1 during performance of the task.

In the second group of experiments we investigate the effect of transcranial random noise stimulation (tRNS). Only one study so far has implemented noisy galvanic stimulation at a very low frequency (< 2 Hz) range targeting the vestibular nerves of patients with levodopa-responsive and unresponsive Parkinsonism over 24 hours (Yamamoto et al., 2005). Here the authors assumed similar effects via the vestibular nerve as otherwise seen with invasive vagal nerve stimulation, for example in patients with epilepsy.

In our experiment we demonstrate this method of enhancing cortico-spinal and cortico-cortical excitability, as measured by TMS, by applying weak motor cortex tRNS for 10 minutes. Furthermore, a variant of the SRTT was used to study tRNS-driven changes in performance (Nissen and Bullemer, 1987). In addition, we show how a mental or motor activity performed during stimulation can reduce the efficacy of tRNS, as previously described in the case of tDCS (Antal et al., 2007).

Methods and Materials

Subjects

Altogether 48 subjects (23 male) participated in the tACS study, and 80 healthy volunteers (32 male) were informed about all aspects of the tRNS experiment. None of the subjects suffered from any neurological and psychological disorders, and none had metallic implants/implanted electric devices, nor took any medication regularly. None of the subjects was on regular or acute medication. All subjects were right-handed, according to the Edinburgh handedness inventory (Oldfield, 1971). We conformed to the Declaration of Helsinki and the experimental protocol was approved by the Ethics Committee of the University of Göttingen.

Experiment 1

1. Transcranial alternating current stimulation (tACS)

8 healthy subjects (22-43 years old, mean age=28.13±8.15, 3 male) participated in the TMS study. 8 healthy subjects (22-32 years old, mean age=25.75±3.28, 3 male) were involved in the EEG experiments. 2 subjects participated in both the EEG and MEP experiments. 13 volunteers (22-31 years old, mean age=24.36±4.15, 6 male) took part in the implicit learning study.

2. Transcranial sinusoidal direct current stimulation (tSDCS)

10 healthy subjects (23-30 years old, mean age=28,7±7.0, 6 men) were involved in the TMS study and 11 subjects took part in the EEG experiments (22-43 years old, mean age=26.8±5.7, 5 male).

Experiment 2

80 healthy volunteers (32 men and 48 women; mean age, 25.74 ± 5.13 years; age range, 20–44 years) participated in the tRNS experiment. Altogether 47 healthy subjects (motor cortex: 17 participants; 21-27 years old; mean age= 23.71 ± 2.08; 6 male; low-frequency/high-frequency: 12 participants; 20-28 years old; mean age= 23.83 ± 3.28; 7 male; DC-shift induced excitability changes: 8 participants; 22-38 years old; mean age= 25 ± 5.12; 4 male; premotor cortex: 10 subjects; 22-39 years old; mean age= 26.5 ± 6.31; 4 male) participated in the single-pulse TMS study. 10 healthy subjects (22-44 years old; mean age= 27.6 ± 6.67; 3 male) were

involved in the paired-pulse TMS experiments, 4 subjects participated in both single- and paired-pulse MEP experiments. 17 volunteers (22-31 years old; mean age= 25.29 ± 2.89; 8 male) took part in the implicit learning study. 12 subjects were involved in the task-related modulation study (22-44 years old; mean age=26.75 ± 6.08; 4 male).

tACS, tSDCS and tRNS

Electrical stimulation was delivered by a battery-driven constant-current stimulator (NeuroConn GmbH, Ilmenau, Germany) through conductive-rubber electrodes, encased in two saline-soaked sponges. In the stimulation mode "noise" there is a random level of current generated for every sample (sampling rate 1280 sps). The random numbers are normally distributed; the probability density function follows a bell-shaped curve. In the frequency spectrum all coefficients have a similar size ("white noise"). The noise signal contains all frequencies up to half of the sampling rate, i.e. a maximum of 640 Hz (Fig.1). In a second experiment this frequency spectrum was separated into a low (0.1 Hz – 100 Hz) and high (101 Hz – 640 Hz) frequency spectrum. Due to the statistical characteristics the signal has no DC offset, provided that the offset is set to zero.

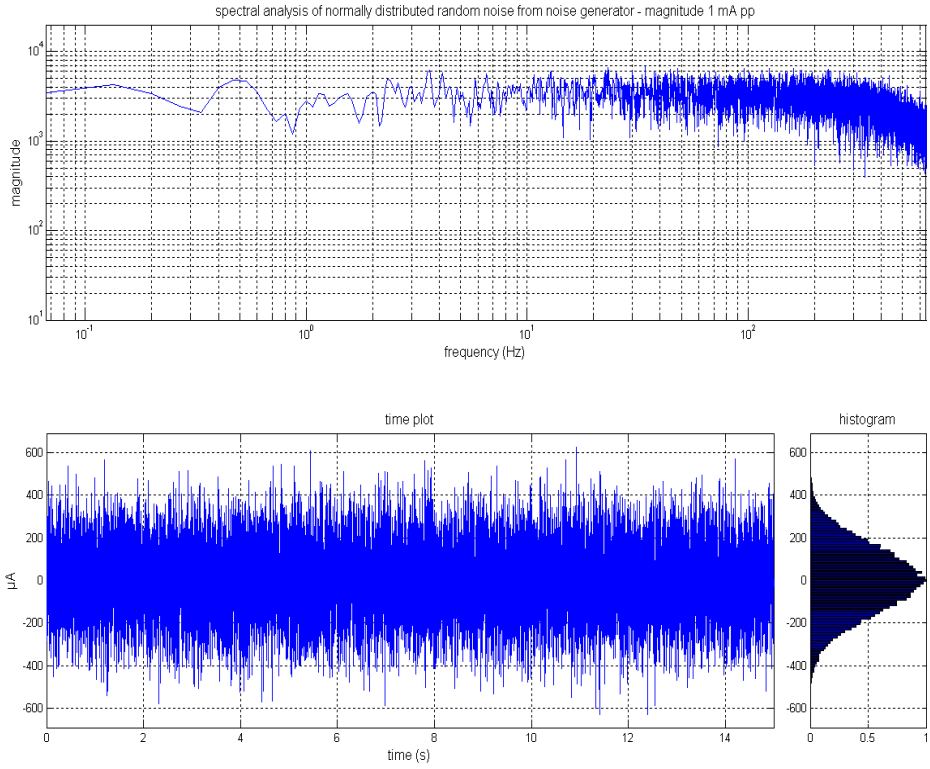
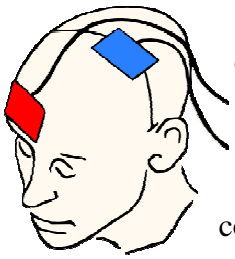


Figure 1. The figure shows the output signal of the DC-Stimulator PLUS, as a frequency distribution of the signal; the time plot of the signal and as a histogram. The signal was generated by a computer. In the stimulation mode "noise" there is a random level of current generated for every sample (sampling rate 1280 sps). The random numbers are normally distributed; the probability density function follows a bell-shaped curve. The amplitude of 1mA pp means that 99% of all generated amplitude values were between +500µA and -500µA.



The stimulation electrode was placed over the left M1, which was determined using single pulse TMS. During the premotor single-pulse TMS study, the stimulation electrode was placed over the premotor cortex (2.5 cm anterior from the M1). To identify the primary motor and premotor cortices the same method was used as that implemented in previous TMS and tDCS studies (e.g. Fink et al., 1997; Munchau et al., 2002). The reference electrode was placed in a saline–soaked sponge over the contralateral orbit. The size of the stimulation electrode was 4x4 cm and the reference electrode was 5x10 cm (tACS, tSDCS) or 6x14 cm (tRNS). The electrodes were fixed by elastic bands.

Experiment 1

TACS was applied for 5 min with a current strength of 400 µA and tSDCS for 2 or 4 min with a current strength of 250 µA. Concerning tSDCS, the AC stimulation was combined with an anodal or cathodal DC shift. In the SRTT study the current was delivered during blocks 2-5, which lasted approximately 7 min. The current was always ramped up or down over the first and last 2 s of stimulation. The maximal current density was 25µA/cm^2 in the case of tACS, and 15.625 µA/cm^2 in the tSDCS experiments, when applied over the M1, which is below the safety parameters accepted for tDCS (Nitsche et al., 2003b). The current density was 8µA/cm^2 or 5µA/cm^2 concerning the reference electrode.

Experiment 2

TRNS was applied for 10 minutes with a current strength of 1000 µA. The maximal current density was 62.5 µA/cm^2 over the M1, which is below the safety parameters accepted for tDCS (Nitsche et al., 2003b). The current density was 12 µA/cm^2 concerning the reference electrode. A supplementary experiment was performed to compare the efficacy of tRNS with that of anodal tDCS. Anodal tDCS was delivered over the left M1 (reference at contralateral orbit) by a battery-driven electrical stimulator (NeuroConn GmbH, Ilmenau, Germany) through

conductive-rubber electrodes, placed in two saline-soaked sponges for 10 minutes with an intensity of 1 mA (Nitsche and Paulus, 2000, 2001).

Subjects were blinded for stimulation conditions in all of the studies. In the case of tACS the TMS-study was double-blind. Subjects were seated in a comfortable reclining chair with a mounted headrest during the experiments. Within each type of experimental session the measurements were always performed by the same investigator.

I. Electrophysiological studies

Transcranial magnetic stimulation (TMS)

To detect current-driven changes of excitability, MEPs of the right first dorsal interosseus muscle (FDI) were recorded following stimulation of its motor-cortical representational field by single-pulse TMS (Fig. 2). These were induced using a Magstim 200 magnetic stimulator (Magstim Company, Whiteland, Wales, UK), with a figure-of-eight standard double magnetic coil (diameter of one winding, 70 mm; peak magnetic field, 2.2 T; average inductance, 16.35 μH). The coil was connected to two monophasic Magstim 200 stimulators via a bistim module (Magstim Co., Whiteland, Dyfed, UK) during the paired-pulse TMS study. Surface electromyogram (EMG) was recorded from the right FDI through a pair of Ag-AgCl surface electrodes in a belly-tendon montage. Raw signals were amplified, band-pass filtered (2Hz-3kHz; sampling rate, 5kHz), digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK) controlled by Signal Software (Cambridge Electronic Design, version 2.13), and stored on a personal computer for off-line analysis. Whenever necessary, complete relaxation was controlled through auditory and visual feedback of EMG activity. The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline, resulting in a posterior-anterior direction of current flow in the brain. This orientation of the induced electrical field is thought to be optimal for the predominantly transsynaptic mode of activation of the corticospinal system. The optimum position was defined as the site where TMS resulted consistently in the largest MEP in the resting muscle. The site was marked with a skin marker to ensure that the coil was held in the correct position throughout the experiment.

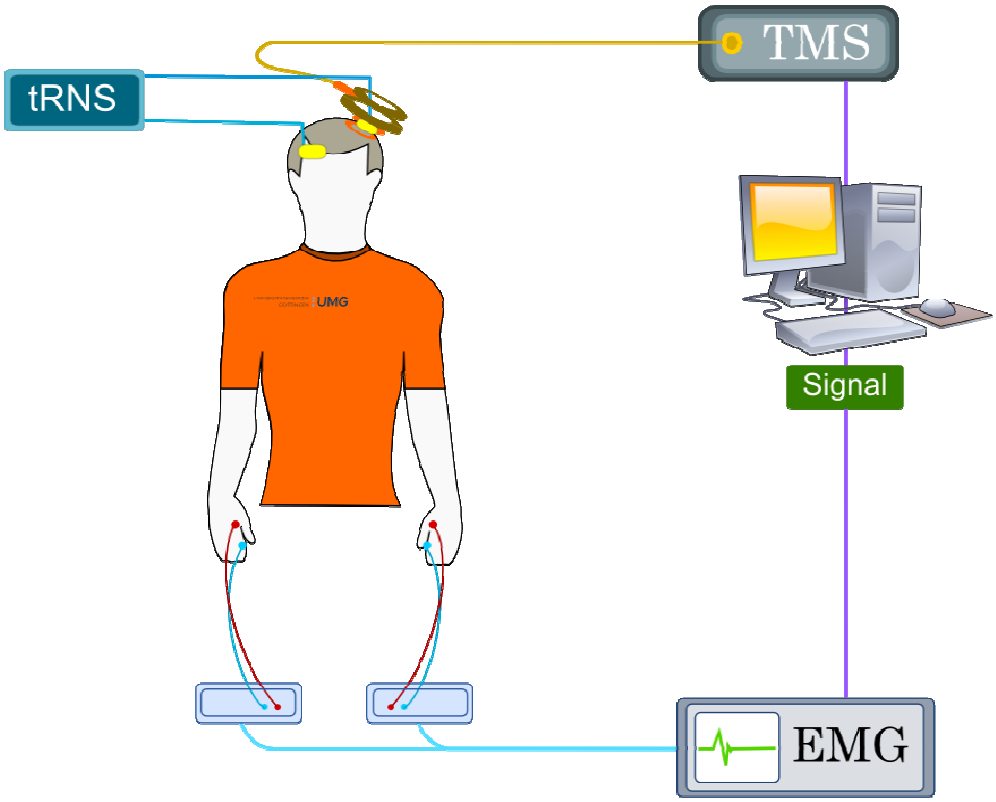


Figure 2. Methods and materials. MEPs of the right FDI muscle were recorded following stimulation of its motor-cortical representational field by single-pulse TMS. These were induced using a Magstim 200 magnetic stimulator, with a figure-of-eight standard double magnetic coil. Surface EMG was recorded from the right FDI through a pair of Ag-AgCl surface electrodes. Raw signals were amplified, band-pass filtered, digitized with a micro 1401 AD converter, controlled by Signal Software.

II. Behavioural studies

Serial Reaction Time Task (SRTT)

A behavioural task was used to study tRNS-driven changes in performance during a variant of the SRTT (Nissen and Bullemer, 1987), which is a standard paradigm to test implicit motor learning. Subjects were seated in front of a computer screen at eye level behind a response pad with four buttons numbered 1-4 and were instructed to push each button with a different finger of the right hand (index finger for Button 1, middle finger for Button 2, ring finger for Button 3, and little finger for Button 4). An asterisk appeared in one of four positions that were horizontally spaced on a computer screen and permanently marked by dots. The subjects were instructed to press the key corresponding to the position of the asterisk as fast as possible. After a button was pushed, the go signal disappeared. The next go signal was

displayed 500 msec later. The test consisted of eight blocks of 120 trials. In blocks 1 and 6, the sequence of asterisks followed a pseudorandom order in that asterisks were presented with equal frequently in each position and never in the same position in two subsequent trials. In blocks 2 to 5 and 7 and 8, the same 12-trial sequence of asterisk positions repeated itself 10 times (abadbcdacbdc). Subjects were not informed about the repeating sequence. Whereas improved performance during the whole course of the task is due to implicit learning as well as to increasing task routine, differences in performance between block 5 and the random block 6 represent a measure of implicit learning only, as task routine is thought to be equivalent in both blocks, and thus any differences in performance should be due to implicit sequence learning (Pascual-Leone et al., 1994).

Task-related modulation of tRNS

In this experiment we showed how a mental or motor activity performed during stimulation can reduce the efficacy of tRNS, as previously described in the case of tDCS (Antal et al., 2007).

III. Safety aspects

All of the subjects completed a questionnaire on the next day after the experimental sessions. The questionnaire contained rating scales for the presence and severity of headache, difficulties in concentrating, acute mood changes, visual perceptual changes, fatigue and discomforting sensations like pain, tingling, itching or burning under the electrodes during and after stimulation.

EEG recording

The EEG was recorded using a three channel montage. One electrode was placed over Oz and two laterally above the motor region (C3 and C4) in accordance with the international 10/20 system. The impedance was kept below 5 kOhm. Linked mastoids (RLm) were used as references; the ground electrode was positioned on the forehead. Data were collected with a sampling rate of 1000 Hz using BrainAmp system (Brain Products GmbH, Munich, Germany) and were analyzed off-line (Brain Vision Analyzer, Brain Products GmbH, Munich, Germany).

Neuron-specific enolase (NSE) determination

To assess the safety of tRNS, we measured serum NSE, a sensitive marker of neuronal damage, evident in many neurological disorders, e.g. in epilepsy (Steinhoff et al., 1999). Elevated NSE concentration is a specific marker in intractable temporal lobe epilepsy.

Experimental design

I. Electrophysiological studies

TMS study

Stimulus intensities (in percentage of maximal stimulator output) of TMS were determined at the beginning of each experiment. Resting motor threshold (RMT) was defined as the minimal output of the stimulator that induced a reliable MEP (~50 μV in amplitude) in at least three of six consecutive trials when the FDI muscle was completely relaxed. Active motor threshold (AMT) was defined as the lowest stimulus intensity at which three of six consecutive stimuli elicited reliable MEPs (~200 μV in amplitude) in the tonically contracting FDI muscle (Rothwell et al., 1999). The intensity of the stimulator output for the single test-pulse MEP was adjusted so that TMS led to an average MEP amplitude of about 1 mV peak-to-peak (SI1mV) before the electrical stimulation. The intensity used to evoke a MEP of SI1mV was used both before and after the AC stimulation.

Experiment 1

1.1 tACS

8 subjects participated in 6 experimental sessions on separate days, one day apart to avoid carry over effects. The TMS experiments were performed at identical times. The subjects received 1, 10, 15, 30 and 45 Hz tACS and sham stimulation in a randomised order. 30 single test-pulse MEPs were recorded seven times after the stimulation, i.e. approximately 0 min after tACS, 2 min, 4 min, 7 min, 10 min, 15 min and 20 min after the end of AC stimulation.

1.2 tSDCS

10 subjects received anodal and 7 cathodal tsDCS with a frequency of 5, 10 and 15 Hz for 2 minutes in a counterbalanced order. Stimulations were done on separate days, and

between each session was at least a 15 min break. 50 single-test pulse MEPs were recorded before and 40 MEPs after tSDCS (averaged in 20 blocks).

Experiment 2

2.1 Single-pulse TMS

a., Motor cortex stimulation

17 subjects participated in 2 experimental sessions, on separate days, at least 3 days apart to avoid carry over effects. The subjects received RN and sham stimulation in a randomised order. Following stimulation, 40 single test-pulse MEPs were recorded at 0.25 Hz, i.e. approximately 0 min, 5 min, 10 min post-stimulation and then every 10 minutes up to 60 min.

Additionally, 8 subjects underwent the same single-pulse TMS experiment (as described previously) in order to investigate the length of the aftereffect of the stimulation. Subjects were measured 0 min, 5 min, 10 min then every 10 minutes up to 60 minutes, then twice in the second hour, then 4 hours, 6 hours and 24 hours post-stimulation. Both active and sham stimulation conditions were applied.

In a second sham-controlled experiment the random noise frequency was divided into a low (0.1 Hz – 100 Hz) and high (101 Hz – 640 Hz) frequency spectrum. 12 participants underwent the same protocol as previously described.

In order to measure DC-shift induced excitability changes, 8 subjects underwent the same protocol as previously described, where the standard DC electrode montage was used (active electrode: anodal - reference electrode: cathodal), and then the electrode montage was reversed (cathodal - anodal).

Furthermore 7 subjects underwent an additional experiment to compare the efficacy of tRNS compared to that of anodal tDCS. With regard to the measurements of MEPs, the same single-pulse TMS protocol was used as previously described.

b., Premotor cortex stimulation

10 subjects participated in 2 experimental sessions on separate days, at least 3 days apart to avoid carry over effects. The subjects received RN and sham stimulation in a randomised order. The study protocol was performed as previously described.

2.2 Paired-pulse TMS

TMS measurements included RMT, AMT and SI1mV, short-interval intracortical inhibition (SICI)/intracortical facilitation (ICF), long-interval intracortical inhibition (LICI), recruitment curves, and cortical silent period (CSP).

10 subjects participated in 4 experimental sessions (1. tRNS: recruitment curves and SICI/ICF; 2. tRNS: LICI and CSP; 3. sham: recruitment curves and SICI/ICF; 4. sham: LICI and CSP) on separate days, at least 3 days apart to avoid carry over effects. The subjects received RN and sham stimulation in a randomised order. Stimulus intensities (in percentage of maximal stimulator output) of TMS were determined at the beginning of each experiment. SI1mV was determined with single-pulse TMS first. RMT and AMT were defined as previously mentioned.

SICI/ICF and LICI were measured with two different protocols of single- and paired-pulse TMS applied in a random order at 0.25 Hz. For SICI/ICF, two magnetic stimuli were given through the same stimulating coil, and the effect of the first (conditioning) stimulus on the second (test) stimulus was investigated (Kujirai et al., 1993). To avoid any floor or ceiling effect, the intensity of the conditioning stimulus was set to a relatively low value of 80% of the AMT. The test-stimulus intensity was adjusted to SI1mV. SICI was measured with interstimulus intervals (ISI) of 2 ms and 4ms, and ICF with ISIs of 9 ms, 12 ms, 15 ms and 25 ms. The control condition (test pulse alone) was tested 40 times, and each of the conditioning-test stimuli 20 times. The mean peak-to-peak amplitude of the conditioned MEP at each ISI was expressed as a percentage of the mean peak-to-peak size of the unconditioned test pulse. The second protocol tested LICI with two suprathreshold stimuli applied with ISIs of 50, 100, 150 and 200 ms (Valls-Sole et al., 1992). The intensity of both stimuli was set to 110% of the RMT. Here as well, the intensity was set to this relatively low value to avoid any floor or ceiling effect. The control condition (first pulse alone) was tested 40 times, whereas each of the paired stimuli was tested 20 times. LICI was taken as the mean percentage inhibition of conditioned MEP at ISIs of 50, 100, 150 and 200 ms.

Recruitment curves were measured with three different and increasing stimulus intensities (110%, 130% and 150% of RMT), each with 10 pulses. A mean was calculated for

all intensities. Finally, 10 pulses with SI1mV and 10 pulses with 120% RMT were applied under tonic contraction of the right FDI muscle. CSPs were separately determined, in rectified and averaged EMG traces with a prestimulus period of 100 ms. CSP (in ms) was measured from the onset of the TMS stimulus to the point where the signal reached the amplitude of the mean prestimulus EMG activity again for >5 ms.

2.3 Intermittent theta burst stimulation (iTBS)

4 subjects, who participated in the single pulse TMS study, underwent an additional experiment to compare the efficacies of tRNS and rTMS. The same single-pulse TMS protocol was used as previously described, with the exception of iTBS, which was applied as an interventional stimulation over the M1. RTMS was delivered using a Magstim Super Rapid stimulator. The pattern of rTMS consisted of bursts containing 3 pulses at 50 Hz, at an intensity of 80% of the predetermined AMT repeated at 200 ms intervals (i.e., at 5 Hz). A 2s train of TBS was repeated every 10s for a total of 190 s (600 pulses) (Huang et al., 2005).

II. Behavioural studies

Serial Reaction Time Task (SRTT)

13 volunteers (tACS) and 17 participants (tRNS) were involved in the implicit learning studies. In the latter case, 6 subjects repeated the first three blocks of the previously used test one (block 9: pseudorandom; block 10-11 repeated sequences) and two hours (block 12: pseudorandom; blocks 13-14: repeated sequences) post-stimulation. Differences in performance between blocks 9-10 and 12-13 also represent a measure of implicit learning. The current was delivered during blocks 2-5, which lasted approximately 7 min. The order of verum and sham stimulation was randomised. The current was always ramped up or down over the first and last 2 s of stimulation.

Task-related modulation of tRNS

The 3 experimental sessions were conducted in a repeated measurement design using a randomized order, with a break of at least 3 days between each session. First, the left motor-cortical representational field of the right FDI was identified using TMS. After determining the

resting and active motor thresholds, a baseline of TMS-evoked MEPs (25 stimuli) was recorded at 0.25 Hz. Afterwards, one stimulation electrode was fixed over the representational field of the right FDI and the other at the contralateral forehead above the orbita.

During tRNS, subjects were passively sitting throughout the stimulation (Experiment 1), had their attention directed towards a cognitive test (Experiment 2) or were instructed to push a ball in their right hand (Experiment 3). After termination of RNS, 25 MEPs were recorded every fifth minute up to 30 min and then every 15 min up to 2 hours.

During the stimulation in Experiment 2, the subjects were required to fill out a cognitive test that was displayed on a computer monitor. The subjects had to push a suitable button with their right index finger in order to give the correct answer. The test was presented in German and downloaded from a commercial intelligence test homepage. The questions were on a variety of subjects. In experiment 3, the subjects were instructed to push a ball (8 cm diameter) in their right hand. The ball was connected to a display where the actual values related to pressure were quantified. Prior to the stimulation session the subjects were asked to push the ball as hard as possible. During the tRNS session subjects had to push the ball to half-maximal contraction as previously shown.

III. Safety

Neuron-specific enolase (NSE) determination

A blood sample for NSE-measurement was taken in 6 healthy subjects before tRNS and 10 min post-stimulation. Furthermore, in 1 subject, who was stimulated on 8 consecutive days, this measurement was performed on every day.

EEG study

Experiment 1
1. tACS

The EEG experiments were conducted in a repeated measurement design, in a randomized order, with a minimum break of 20 minutes between each stimulation session. Two minutes EEG was recorded at rest before, and 3 times after AC stimulation (immediately, 7

minutes and 14 minutes after the end of the stimulation). Subjects received 1, 10 and 45 Hz tACS in a randomised and counterbalanced order.

2. *tSDCS*

tSDCS was administered at 5, 10 and 15 Hz in a randomised order, with a 20 minute break between stimulation sessions. A 2 minute EEG was recorded prior to stimulation, and then a 4 minute EEG recorded immediately post-stimulation. Subjects received tSDC for a 4-minute-duration, at an intensity of 250 μA in both an anodal and cathodal direction.

Experiment 2

The EEG experiments were conducted in a repeated measurement design (tRNS and sham) using a randomized order, with a minimum break of 1 day between each stimulation session. 2 minutes EEG was recorded at rest before, and three times after stimulation (immediately, 7, and 14 minutes after the end of the stimulation).

For sham stimulation the current was turned on for 8 seconds at the beginning of the stimulation in order to achieve the light itching sensation under the electrode. Subjects were blinded for stimulation conditions in all of the studies.

Data analyses

I. Electrophysiological studies

Peak-to-peak amplitudes (mV) of each MEP were measured off-line, and mean MEP amplitudes were calculated for each stimulation condition, at each time point separately.

1. Single-pulse TMS

Repeated measurements of ANOVAs (CONDITION (tACS/tSDCS or tRNS vs. sham) x TIME (before; 0, 5, 10, 20, 30, 40, 50, 60 min post-stimulation; (24 hours after measurement (n=8): before; 0, 5, 10, 20, 30, 40, 50, 60, 90 min and 2, 4, 6, 24 hours post-stimulation) were used to compare the different conditions. Effects were considered significant if $p<0.05$. In the case of a significant interaction of TIME and stimulation CONDITION, a Tukey post-hoc test was performed. Student´s t-test was used to compare the motor thresholds (RMT, AMT and SI1mV) between experimental sessions. All data are given as means + SEM.

2. Paired-pulse TMS

For each measurement (SI1mV, RMT, AMT, SICI/ICF, LICI, CSP), we performed separate analyses of variance (ANOVAs) for repeated measurements, by using the mean values from each subject as the dependent variable. In addition to the factor STIMULATION type (tRNS vs. sham), the ANOVA model included the factor ISI (2, 4, 7, 9, 12, 15 and 25 ms) when SICI/ICF was analysed, or the factor INTENSITY (100%, 130%, and 150% of RMT) for recruitment curves, or the factor INTENSITY (120% RMT and SI1mV) for CSP. A p value of <0.05 was considered significant for all statistical analyses. As the differences between the values of SICI/ICF might not be detectable with ANOVA, additional Student's t-tests for dependent variables were performed to compare the differences between the tRNS and sham conditions at all of the different ISIs, separately. Student´s t-test was used to compare the motor thresholds (RMT, AMT and SI1mV) between experimental sessions. Data are expressed as mean ± SEM.

II. Behavioural studies

SRTT analysis

Concerning the implicit learning paradigm, statistical analysis was performed with repetitive measures ANOVA (independent variables current CONDITION and BLOCK) for reaction time (RT), error rate (ER), and variability. As the RT and ER differences between blocks 5 and 6 are thought to represent an exclusive measure of implicit learning, interactive Student's t-tests were performed to compare the respective differences between tACS/tRNS and sham conditions. In each trial, RT was measured from the appearance of the "go" signal until the first button was pushed by the subject. For each block of trials of a given experimental condition, mean RT was calculated for each subject separately. Furthermore, the standard error of RTs for each subject in every block was calculated as an index of variability of RTs. An ER was calculated to assess the number of incorrect responses for each block and each subject in each stimulation condition.

Task related modulation of tRNS

Repeated measures ANOVA (EXPERIMENT (passive vs. cognitive/motor) x TIME (before, 5, 10, 15, 20, 25, 30, then every 15 min up to 2 hours) was used to compare different task conditions during tRNS. Effects were considered significant if $p < 0.05$. In case of a significant interaction of time and stimulation condition, a Tukey post-hoc test was performed. Student´s t-test was used to compare the motor thresholds (RMT, AMT and SI1mV) between experimental sessions.

III. Safety

NSE-determination

Two-tailed t-tests (paired samples, critical p-value 0.05) were performed to compare NSE-values before and after tRNS.

EEG recording

EEG epochs (2 min) were segmented for 30 seconds and filtered using 0.1 Hz (24 dB/octave) low cutoff, 70 Hz (24 dB/octave) high cutoff, and 50 Hz notch filters. In addition to semiautomatic artefact detection (200 µV amplitude criterion) all epochs were visually inspected, and those containing eye blinks or muscle movement artefacts were excluded. After artefact rejection all of the epochs were segmented into 2 s and Fast Fourier Transformation (FFT) was calculated for all electrodes (0.5 Hz resolution, and 10% Hamming-window). The FFT segments were averaged for each 30 s. The mean activity in voltage was calculated and exported for each frequency band (theta band 4.5-7 Hz, alpha band 8-12 Hz, beta band 12.5-30 Hz and gamma band 31-49 Hz) for statistical analysis. In order to compare the effect of stimulation on the EEG spectrum, a repeated measures ANOVA (independent variable: tACS/tRNS vs sham x time points of post-stimulation; dependent variable: FFT power in a given frequency band) was calculated.

Results

All of the subjects tolerated the stimulation; none of the experimental sessions were interrupted due to any side effects of the stimulation. However, about half of the subjects noticed a flickering light in their visual field, during higher frequency tACS using an intensity of 0.4 mA. Consequently, we did not increase the stimulation amplitude any further for safety reasons. Only 2 of the subjects reported a light burning sensation under the electrodes. 6 subjects had light headache after the tACS session. In the case of tRNS only 2 out of 80 subjects reported a slight burning sensation under the electrodes during the stimulation.

I. Electrophysiological studies - MEPs

Experiment 1

1. tACS

The repeated measurements of ANOVA revealed no significant interactions between current CONDITION and TIME, in any of the cases comparing tACS and sham stimulation ($F<1.0$, $p>0.2$). A marked decrease of motor-cortical excitability after 10 Hz stimulation, of approximately 20% ($p=0.08$) was observed. All other stimulation frequencies (1, 15, 30, 45 Hz) were ineffectual in inducing aftereffects. Table 2 shows the mean MEP values and their standard errors before and after tACS.

	1 Hz	10 Hz	15 Hz	30 Hz	45 Hz	sham
Before	1.02 ± 0.11	1.03 ± 0.13	1.03 ± 0.09	1.03 ± 0.08	1.04 ± 0.09	1.02 ± 0.11
0 min	1.01 ± 0.30	0.93 ± 0.31	1.15 ± 0.37	1.06 ± 0.33	1.15 ± 0.46	1.19 ± 0.42
2 min	1.04 ± 0.44	0.94 ± 0.31	1.05 ± 0.41	1.11 ± 0.38	1.11 ± 0.47	1.20 ± 0.38
4 min	1.16 ± 0.37	0.91 ± 0.37	1.17 ± 0.34	1.16 ± 0.33	1.30 ± 0.51	1.20 ± 0.31
8 min	1.14 ± 0.35	0.92 ± 0.43	0.98 ± 0.27	1.15 ± 0.29	1.19 ± 0.45	1.20 ± 0.36
10 min	1.20 ± 0.45	0.99 ± 0.36	1.13 ± 0.37	1.14 ± 0.29	1.06 ± 0.51	1.31 ± 0.46
15 min	1.32 ± 0.53	1.08 ± 0.40	1.13 ± 0.27	1.20 ± 0.20	1.09 ± 0.41	1.16 ± 0.41
20 min	1.27 ± 0.52	0.99 ± 0.27	1.21 ± 0.20	1.11 ± 0.33	1.06 ± 0.43	1.04 ± 0.22

Table 2. Mean MEP amplitudes (and their SEMs) before and after tACS. A marked decrease of the MEP amplitude after 10 Hz stimulation was observed, however, it was not significant.

2. tSDCS

Here, AC stimulation at a given frequency was combined with a DC shift in an anodal or cathodal direction. The ANOVA revealed no significant interactions between current CONDITION and TIME for either the anodal or cathodal condition ($F<1.2$, $p>0.3$). A marked increase in motor-cortical excitability after the combination of anodal and 15 Hz stimulation, of approximately 40% was observed after stimulation. However, this increase was not significant compared to baseline values ($p=0.08$). Table 3 shows the mean MEP values and their standard errors before and after tSDCS.

	Anodal (mean MEPs and SEM)			**Cathodal (mean MEPs and SEM)**		
	before	**2 min after**	**4 min after**	**before**	**2 min after**	**4 min after**
5 Hz	1,12 ± 0,1	1,12 ± 0,2	1,16 ± 0,2	0,92 ± 0,06	1,08 ± 0,14	0,8 ± 0,15
10 Hz	1,04 ± 0,08	1,27 ± 0,1	1,13 ± 0,1	0,97 ± 0,06	0,92 ± 0,11	0,94 ± 0,16
15 Hz	1,2 ± 0,03	1,6 ± 0,2	1,37 ± 0,11	0,89 ± 0,1	1,08 ± 0,2	0,9 ± 0,2

Table 3. Mean MEP amplitudes (and their SEMs) before and after tSDCS at 5, 10 and 15 Hz stimulation. A marked increase in the MEP amplitude after anodal 15 Hz stimulation was observed, however, it was not significant.

Experiment 2

1. Single-pulse TMS

When 10 min tRNS was applied over the M1, the induced cortical excitability increases rose up to 20-50%, as revealed by TMS. They lasted for 60 minutes post-stimulation. Repeated measurements of ANOVA revealed a significant main effect of CONDITION ($F(1,28)=7.24$, $p=0.01$) and TIME ($F(8,224)=4.01$, $p<0.001$) in the case of M1 stimulation. The interaction between CONDITION and TIME was also significant ($F(8,224)=3.53$, $p<0.001$) (Table 4). According to the post-hoc analysis, significantly increased MEPs were observed at the 5 and 10-60 min timepoints compared to the timepoint before ($p<0.05$) tRNS (Fig. 3).

				df	**t**	**p**
Single-pulse TMS	**Student's t-test**	**RMT**		10	0.90	0.39
		AMT		10	1.68	0.12
		SI1mV		10	0.42	0.69
	ANOVA		**Factor**	**df**	**F**	**p**
			condition	1	7.24	**0.01**
			time	28	4.01	**<0.01**
			condition x time	28	3.53	**<0.01**
				df	**t**	**p**
Paired-pulse TMS	**Student's t-test**	**RMT**		9	0.42	0.68
		AMT		9	0.90	0.39
		SI1mV		9	0.01	1.00
	ANOVA	**RECR**	**Factor**	**df**	**F**	**p**
			condition	1	0.80	0.39
			intensity	2	19.03	**<0.01**
			condition x intensity	2	0.38	0.69
		SICI/ICF	condition	1	0.14	0.72
			ISI	5	27.55	**<0.01**
			condition x ISI	5	1.85	0.12
		LICI	condition	1	0.23	0.64
			ISI	4	4.04	0.01
			condition x ISI	4	0.37	0.83
		CSP	condition	1	0.63	0.44
			intensity	1	1.05	0.33
			condition x intensity	1	0.81	0.38

Table 4. Results of the statistical analyses in the case of the single- and paired-pulse TMS studies over the M1.

RMT, AMT and SI1mV baseline values were compared for RN and sham stimulation conditions using Student's t-test. There was no significant difference between tRNS and sham stimulation in any of the measurements (Table 4).

Furthermore, we separated the stimulation spectrum into low- (0.1 Hz-100 Hz) and high-frequency ranges (101 Hz-640 Hz). Repeated measurements of ANOVA revealed a marginally significant effect of CONDITION ($F(2,33)=3.02$, $p=0.06$) and a significant effect of TIME ($F(16,264)=2.39$, $p=0.02$). There was no significant CONDITION x TIME interaction ($F(16,264)=1.44.$, $p=0.12$) (Fig. 4).

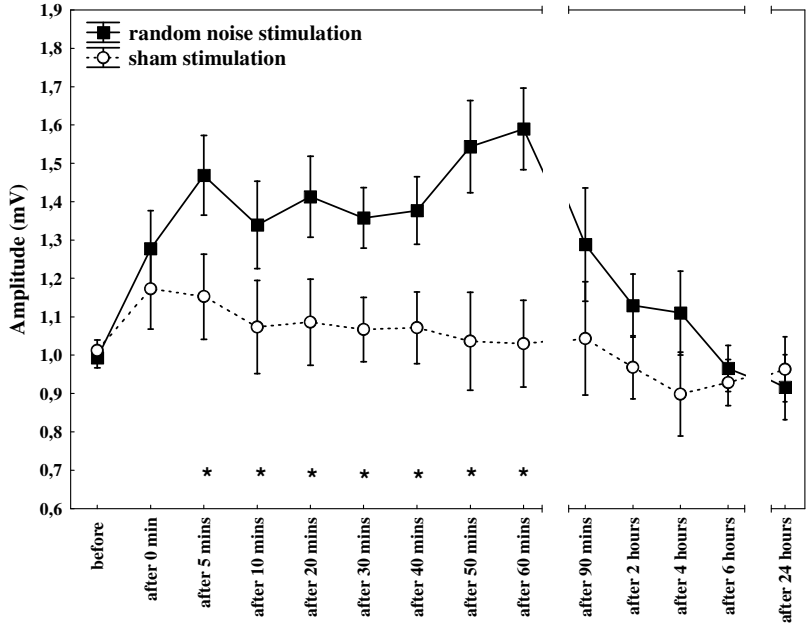


Figure 3. Effect of 10 min RN stimulation on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min RN stimulation over M1 at 1mA, compared to sham stimulation. The figure shows mean amplitudes and their SEMs up to 60 min (including all subjects, n=17) and between 90 min and 24 hours (including eight subjects). Asterisks indicate significant differences between MEP amplitudes after 5, 10-60 min post-stimulation compared to baseline.

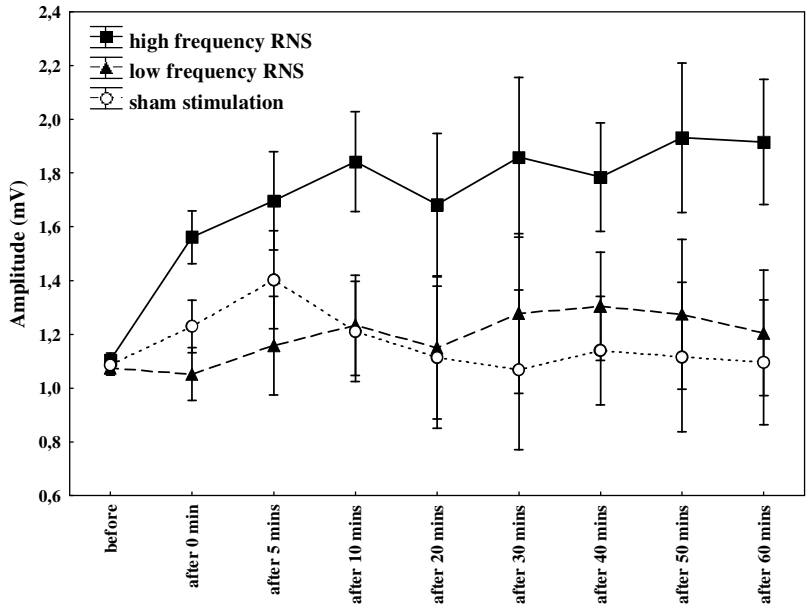


Figure 4. Effect of 10 min low- (0.1 Hz-100 Hz) and high-frequency (101 Hz-640 Hz) RN stimulation on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min high-frequency RN stimulation over M1 at 1mA, compared to low-frequency and sham stimulation. The figure shows mean amplitudes and their SEMs up to 60 min (including all subjects, n=12).

We did not observe any changes in cortico-spinal excitability when the premotor cortex was stimulated, implying that the effect of tRNS over the M1 is indeed focal. Repeated measurements of ANOVA revealed no significant effect of CONDITION ($F(1,18)=0.01$,

p=0.99) nor TIME (F(8,14)=0.78, p=0.61). There was no significant CONDITION x TIME interaction (F(8,14)=0.69, p=0.70).

The possibility of a hidden DC-shift in the stimulation spectrum as a cause of the excitability increase was excluded by a control experiment with reversed electrodes. In the case of measuring DC-shift induced excitability changes, repeated measurements of ANOVA revealed no significant effect of CONDITION (F(1,14)=0.29, p=0.60). The effect of TIME was significant (F(8,112)=2.13, p=0.04). There was no significant CONDITION x TIME interaction (F(8,112)=0.24, p=0.98).

A supplementary experiment was performed to compare the efficacy of tRNS with that of anodal tDCS. Fig. 5 shows the effect of 10 mins RNS on MEPs compared to conventional anodal tDCS after-effects. The time course of M1 excitability change lasts for 60 minutes post-stimulation after tRNS. However, the facilitation of MEP size following tDCS lasts for approximately 40 mins. The figure shows mean amplitudes and their SEMs.

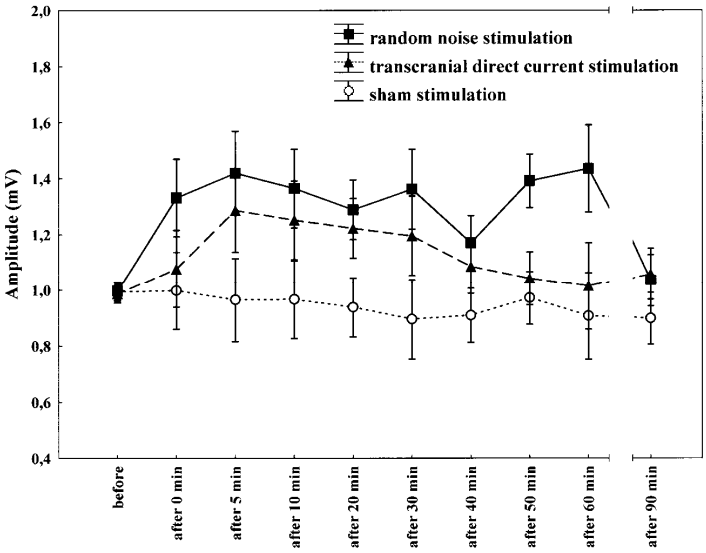


Figure 5. Effect of 10 min tRNS and anodal tDCS on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min RN stimulation over M1. However, the facilitation of MEP size following anodal tDCS lasts for approximately 40 mins. The figure shows mean amplitudes and their SEMs up to 90 min (including all subjects, n=7).

2. Paired-pulse TMS

In our paired-pulse TMS study we have observed an increase in ICF after tRNS over M1. TRNS administration had no effect on SICI, LICI, CSP or motor-evoked recruitment curves as revealed by repeated measurements of ANOVA (Table 4). However, Student's t-tests showed significant differences in the case of ICF with ISIs of 12 ms (t=2.40, df=9, p=0.03) and 25 ms (t=-2.28, df=9, p=0.047) showing an increased facilitation after RNS. This phenomenon

may be explained by the activation of cortico-cortical pyramidal cells and their axons (Ziemann, 1999).

3. Intermittent theta burst stimulation (iTBS)

Fig. 6 shows the effect of 10 mins RN stimulation on motor evoked potentials compared to conventional iTBS after-effects. The time course of M1 excitability change lasts for 60 minutes post-stimulation after tRNS. However, the facilitation of MEP size following iTBS lasts for approximately 30 mins.

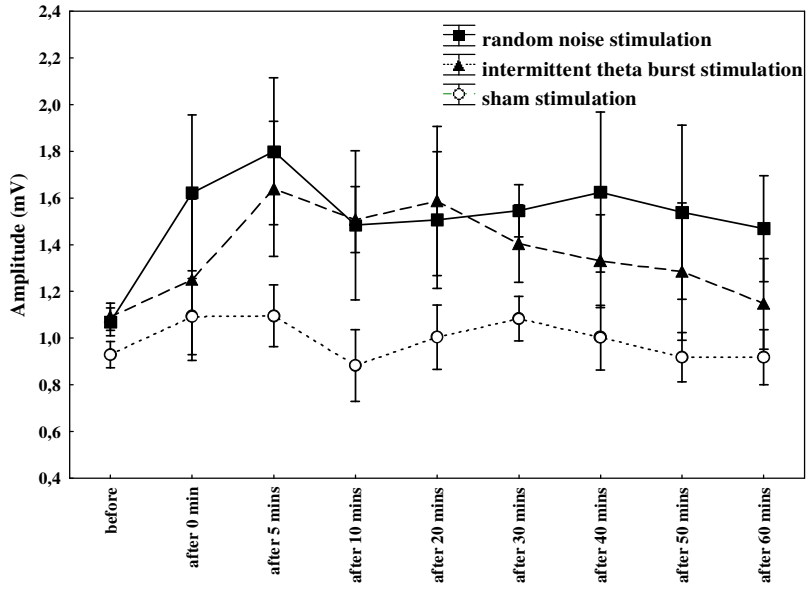


Figure 6. Effect of tRNS and rTMS on motor evoked potentials. The pattern of rTMS consisted of bursts containing 3 pulses at 50 Hz at an intensity of 80% AMT, repeated at 200 ms intervals (i.e., at 5 Hz). 2s train of TBS was repeated every 10s for a total of 190 s (600 pulses). The time course of M1 excitability change lasts for 60 minutes post-stimulation after tRNS over M1 at 1mA. However, the facilitation of MEP size following iTBS lasts for approximately 30 mins. The figure shows mean amplitudes and their SEMs up to 60 min (including all subjects n=4)

II. Behavioural studies

1. SRTT

With regard to the functional effect of tACS and tRNS, they significantly improved performance in the acquisition and early consolidation phase of motor learning. Compared with the sham stimulation condition, RTs in the SRTT shortened during 10 Hz tACS and tRNS of the M1, and subjects became faster during the course of the experiment.

Experiment 1

RTs of the SRTT shortened during tACS of the M1; repeated measures ANOVA revealed a significant effect on BLOCKS ($p<0.001$) at all frequencies. This was caused by an interaction of alternating current versus sham stimulation for block 5 and block 6, due to a greater difference in the alternating current stimulation in the case of 10Hz stimulation ($t=-2.76$, $df=12$, $p=0.017$) as revealed by Student's t-tests. Fig. 7 shows the differences between 10 Hz and sham stimulation. Despite the significant main effect of BLOCKS in ANOVA, the results of all other tests remained insignificant. However, a trend toward reduced RTs in blocks 2-5 and 7 for tACS compared to the sham condition was identified.

For ER, the ANOVAs showed a significant main effect of CONDITION ($p<0.001$) and BLOCKS at 1 Hz ($p=0.012$) and at 45 Hz ($p=0.001$), there was no significant CONDITION X BLOCKS interaction. Student's t-tests revealed no significant difference between blocks 5 and 6. For variability, the ANOVAs showed a significant main effect of CONDITION ($p<0.001$) and BLOCKS ($p<0.001$) without a significant interaction between CONDITION and BLOCKS at all frequencies. Student's t-tests revealed no significant differences between blocks 5 and 6.

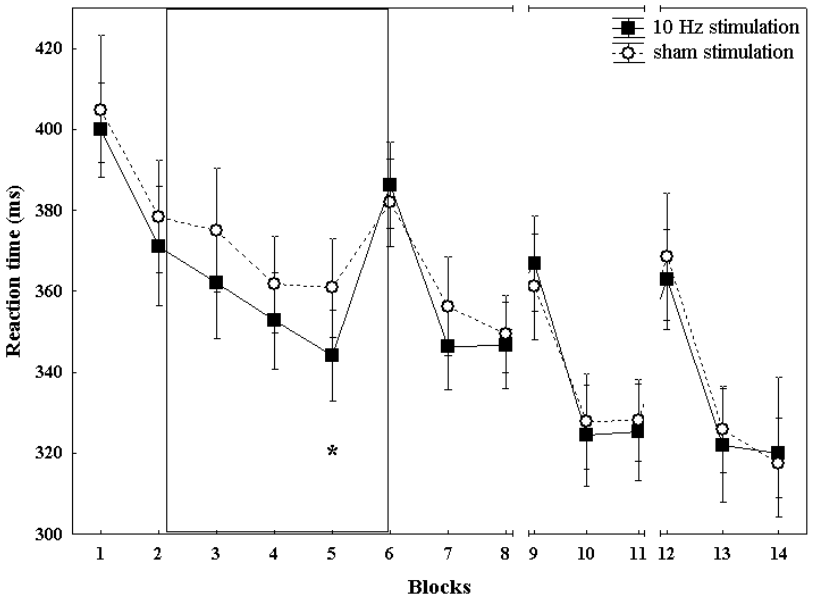
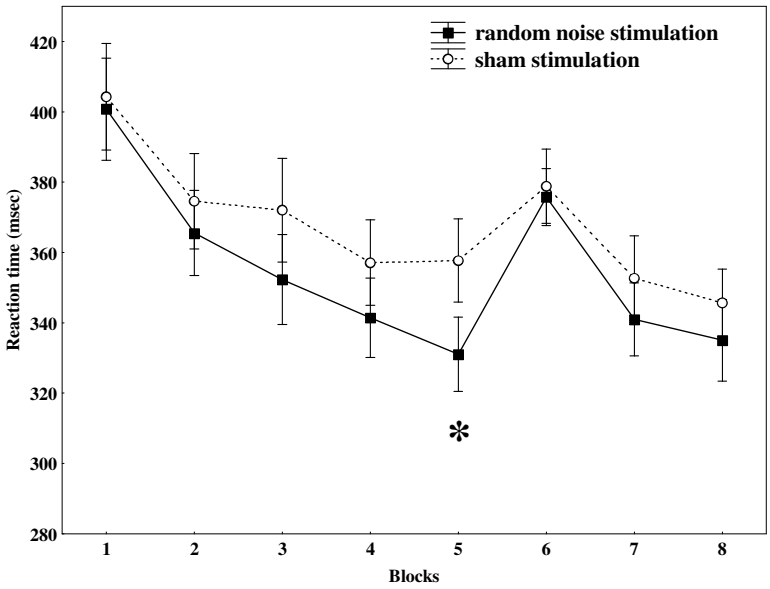
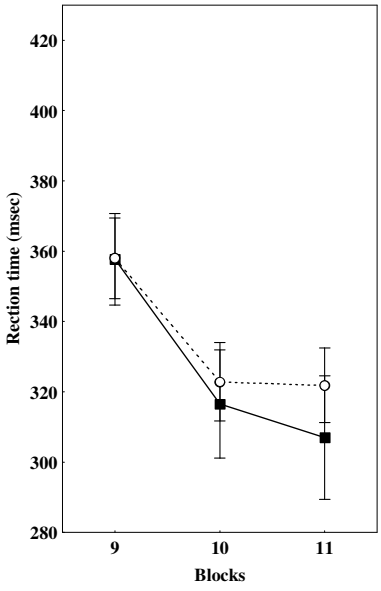


Figure 7. 10 Hz tACS of the M1 improves implicit motor learning in its early phase. Reaction times decrease faster in the 10 Hz stimulation condition compared to the sham stimulation condition. Moreover, the RT difference comparing blocks 5 and 6, which indicates implicit sequence learning most purely, is bigger for the 10 Hz stimulation condition, when compared to the non-stimulation condition. The asterisk shows a significant difference regarding the reaction time differences between blocks 5 and 6, comparing 10 Hz and sham stimulation.

Experiment 2

Repeated measures ANOVA revealed a significant effect on BLOCKS ($F(7,11)=37.59$, $p<0.001$). This was caused by an interaction of tRNS versus sham stimulation for block 5 and block 6, due to a greater difference in the case of tRNS ($t=-2.87$, $df=16$, $p=0.01$) as revealed by Student's t-tests. There was no significant effect on stimulation. However, the CONDITION x BLOCKS interaction was only marginally significant ($F(7,11)=1.95$, $p=0.06$). Fig. 8 shows the differences between RN and sham stimulation. The paradigm was repeated in 6 subjects after one and two hours post-stimulation. At these timepoints the RTs were not significantly different between the tRNS and sham conditions (see Fig. 8).





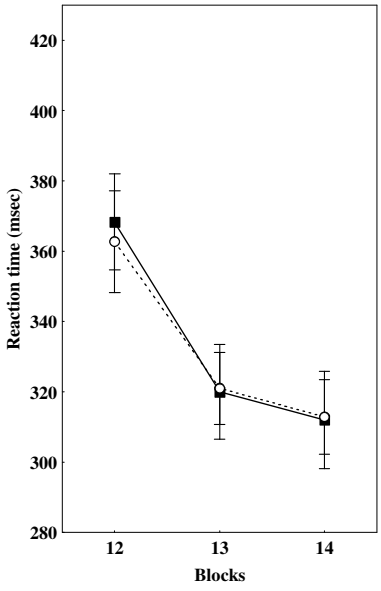


Figure 8. TRNS of the M1 improves implicit motor learning in its early phase. Reaction times decrease faster in the tRNS condition when compared to the sham stimulation condition (upper figure). Moreover, the RT difference comparing blocks 5 and 6, which indicates implicit sequence learning, is bigger for the tRNS condition, when compared to sham condition. The asterisk indicates a significant difference regarding reaction time differences between blocks 5 and 6, between RN and sham stimulation. In one and two hours post-stimulation this significant difference was no longer detectable (lower figures).

For the ER the ANOVAs showed a significant main effect on BLOCKS ($F(7,11)=2.54$, $p=0.02$). Despite this, the results of all other tests remained insignificant. Student's t-tests revealed no significant difference between blocks 5 and 6. For variability, the ANOVAs showed a significant main effect on BLOCKS ($F(7,11)=8.56$, $p<0.001$) without a significant interaction between CONDITION and BLOCKS.

2. Task-related modulation of tRNS

Excitability increase induced by tRNS was modified by paying attention to a task involving mental activity and by contraction of the target muscle during the stimulation. Following tRNS the amplitude of the MEPs was increased in the passive condition, slightly decreased in the cognitive condition and markedly reduced in the motor condition. When the amplitude of the MEPs was compared with regard to the passive condition and cognitive task before and after stimulation, repeated measures ANOVA revealed a main effect of EXPERIMENT ($F(1,11)=5.45$, $p=0.04$) but TIME ($F(12,132)=0.50$, $p=0.91$) was not significant. The interaction between the EXPERIMENT and TIME was significant ($F(12,132)=2.36$, $p=0.009$). The post-hoc test revealed that, after tRNS in the passive condition, significantly increased MEP amplitudes were observed up to 20 mins, and at the 1 and 2 hours timepoints when compared to the cognitive task condition ($p<0.01$). When the amplitude of the MEPs was compared with the passive condition and motor task, repeated measures of ANOVA revealed a main effect of EXPERIMENT ($F(1,11)=10.05$, $p=0.009$) but TIME ($F(12,132)=0.74$, $p=0.71$) was not significant. The interaction between the EXPERIMENT and TIME was significant ($F(12,132)=3.96$, $p<0.001$). The post-hoc test revealed that, after tRNS in the passive condition, significantly increased MEP amplitudes were observed up to 25 mins post-stimulation ($p<0.01$), compared to the motor condition.

III. Safety

1. NSE

The concentration of serum NSE was unchanged after tRNS. Student's t-test showed no significant difference between the before and after stimulation NSE concentrations of 6 healthy subjects (t=0.09, p=0.93, mean value before stimulation:6.96+/-1.84 ug/l, after stimulation: 6.91+/-1.7 ug/l). One subject was stimulated for 10 minutes every day for 8 consecutive days. The NSE values did not change significantly over the stimulation period as measured from the first to last day of stimulation (t=-0.2, p=0.87, mean value before stimulation:9.57+/-2.2 ug/l, after stimulation: 9.53+/-3.0 ug/l).

2. EEG

We recorded EEGs before and after different types of stimulations and did not find any significant difference regarding any frequency bands. Repeated measures ANOVA revealed no significant interactions between current CONDITIONS, TIME or CHANNELS for any of the different frequencies applied. Additionally, we did not see any abnormal EEG activity after tACS/tSDCS or tRNS. Therefore, we can conclude that limited exposure to these stimulations of the cortex, using the parameters we applied here, is safe.

Discussion

The aim of our present studies was to investigate new non-invasive transcranial stimulation techniques. We aim to further expand the stimulation spectrum between DC and AC stimulation. For this we applied a frequency spectrum between 1 and 45 Hz using transcranial electrical stimulation and analysed MEPs, EEG-spectra and behavioural tasks, before and after AC stimulation, with and without an anodal and cathodal DC shift. The main result of this study was that 10 Hz tACS over the M1 using a 7 min stimulation duration was able to improve implicit motor learning, and it modified motor cortical excitability that outlasted the stimulation duration itself (for a summary of our results see Table 5). A marked decrease in MEP amplitude following 10 Hz AC stimulation was observed, compared to sham stimulation, without modifying EEG power. The improved implicit motor learning following AC stimulation is similar to the effect of anodal stimulation over the M1 reported in a previous study (Nitsche et al., 2003a). In our study only 10 Hz tACS improved performance in the acquisition and early consolidation phase of implicit motor learning significantly. Compared to the non-current stimulation condition, reaction times in the SRTT decreased faster significantly, during the course of the experiment. Previous studies suggest that an excitability enhancement seems to be a necessary condition for learning by inducing strengthening of synapses/long-term-potentiation by modifying NMDA-receptor efficacy (Bennet, 2000; Rioult-Pedotti et al., 2000). Regarding studies in the human, this is in line with observations of increased activation of the M1 during motor learning tasks (Grafton et al., 1992; Honda et al., 1998), and also with pharmacological studies showing that the results of motor training can be improved by cortical excitability enhancements (Butefisch et al., 2002). It appears that a 10 Hz tACS-driven cortical excitability change could facilitate the learning process.

However, the marked inhibition observed in the amplitude of MEPs after 10 Hz stimulation, that we have seen in this study, showed a similar pattern to that of cathodal tDCS over the M1, observed in previous studies (Nitsche and Paulus, 2000; 2001). The result, at least at first glance, is surprising, taking into account the fact that using rTMS with a stimulation frequency higher than 1 Hz usually results in a facilitatory effect over the cortex (for a review see Rossi and Rossini, 2004). However, it was also published that there was no significant change in cortico-spinal excitability following 10 Hz rTMS. In our study 10 Hz tACS had an inhibitory effect on MEP amplitudes, but the same stimulation was able to improve motor performance.

	Type of electrical stimulation	Study			Current intensity	Stimulation time	Main result of the experiment
Experiment 1 n=48	**tACS** 1, 10, 15, 30, 45 Hz	Electrophysiological studies	single-pulse TMS		400 µA	5 min	∅
		Behavioural studies	SRTT			~7 min	10 Hz: ↑ performance
		Safety aspects	EEG			4 min	∅
	tSDCS 5, 10, 15 Hz	Electrophysiological studies	TMS		250 µA	2, 4 min	∅
		Safety aspects	EEG			4 min	∅
Experiment 2 n=80	**tRNS**	Electrophysiological studies	single-pulse TMS	motor cortex	1000 µA	10 min	↑
				low frequency/high frequency			∅
				DC-shift induced excitability changes			∅
				premotor cortex			∅
				comparing to anodal tDCS			longer excitability changes after tRNS
			paired-pulse TMS				ICF: ↑
			iTBS				longer excitability changes after tRNS
		Behavioural studies	SRTT			~7 min	↑ performance
			task-related modulation			10 min	modified excitability increase
		Safety aspects	EEG			4 min	∅
			NSE				∅

Table 5. Summarizing table of our experiments.

The only difference between the two tACS studies was the duration of the stimulation: in the TMS study, shorter stimulation duration was applied than that in the implicit learning study. Therefore, it might be possible that the effect of 10 Hz tACS is stimulation duration-dependent; a shorter stimulation duration may have inhibitory effects, whilst a longer duration facilitatory effects.

We used a relatively small stimulation electrode in order to enhance the focality of the stimulation (Nitsche et al., 2007) and a larger reference electrode to avoid stimulation of the frontopolar cortex and retina. However, half of the subjects still noticed a flickering sensation, mainly during high frequency stimulation. Further increase of the reference electrode size technically is not possible, therefore in the future, systematically exploring the effect of electrical stimulation using new electrode positions (e.g. M1 – occipital cortex) is necessary. If intensities are comparable between tDCS and tACS, 4 mA might be the lower border for inducing aftereffects (Nitsche and Paulus, 2000). Thus it remains to be seen whether higher intensities are better for inducing aftereffects, notwithstanding the assumption that they are potentially more dangerous with respect to seizure induction.

A recent study by Kanai et al. (2008) showed that tACS can interact with ongoing rhythmic activities in the visual cortex in a frequency-specific fashion and induce visual phosphenes. Stimulation over the occipital cortex induced perception of continuously flickering light most effectively when the beta frequency range was applied in an illuminated room, whereas the most effective stimulation frequency shifted to the alpha frequency range during testing in the darkness. The authors suggested that the frequency dependency is caused by interactions with ongoing oscillatory activity in the stimulated cortex.

In our second experiment, we investigated a new stimulation technique, namely tRNS. In that study we demonstrated that weak tRNS over M1 enhances cortico-spinal excitability both during and after stimulation in the healthy human brain (Table 5). Furthermore, our results suggest that the high frequency subdivision of the whole tRNS spectrum between 100 and 640 Hz is functionally responsible for inducing excitability in the M1. In terms of commonly used non-invasive excitability parameters, we have shown that this excitability increase is due to an increase in ICF after tRNS over M1 in the paired-pulse study (Table 5). TRNS administration had no effect on SICI, LICI, CSP or motor-evoked recruitment curves (for an overview of available methods for studying the modulation of human motor cortex excitability by local circuits see Paulus et al, 2008; Ziemann et al, 2008). Pharmacological studies show that amongst other neurotransmitter systems, ICF is most likely to be mediated by the glutamatergic

system (Ziemann et al., 1998) compatible with an activation of glutamatergic synapses by tRNS.

The MEP declines observed after mental effort and motor activation are in agreement with previous studies using tDCS (Antal et al., 2007) or paired associative stimulation (PAS) (Stefan et al., 2004). Similarly, a recent study observed that contraction of the FDI muscle during TBS abolished the aftereffects of stimulation on MEPs (Huang et al., 2007). These results suggest that the externally induced neuronal plasticity is highly dependent on the state of the subject during stimulation.

It appears that the tRNS-driven cortical excitability change facilitates the learning process. Additionally, our results describing an increase in cortico-spinal excitability which accompanies the facilitation in learning with regard to the SRTT, more closely resemble those reported by previous studies after anodal tDCS (Nitsche and Paulus 2000, 2001); even more so, since we applied well-proven tDCS parameters such as electrode position, intensity and stimulation duration.

There is however, a key difference between tDCS and tRNS. TDCS modifies the transmembrane neuronal potential directly, and thus modulates the firing rate of individual neurons (Bindman et al., 1964). In contrast, the oscillatory spectrum of tRNS does not have a DC component. Also the physiological control experiment with the reversal of the electrode positions within the DC tested montage did not influence the characteristic excitability enhancing aftereffect, in contrast to the inhibition which we see with cathodal tDCS (Nitsche and Paulus, 2000). Several physiological mechanisms may underlie the observed tRNS effects. TRNS, like alternating current stimulation, can possibly interfere with ongoing oscillations and neuronal activity in the brain and thus result in increases in cortical excitability. However, tACS with intensities higher than 400μA induced a flickering sensation via retinal stimulation and as a result, we were reluctant to increase the intensity further, at least with the standard reference montage at the forehead close to the retina. Also, the tACS type of monophasic sinusoidal stimulation is more likely to be epileptogenic than that of a random noise waveform. For this reason we started by using a random noise frequency spectrum with a range of 0.1 to 640 Hz, the latter frequency known to represent the high end of physiologically measured human electric brain oscillations (Gobbelé et al., 2000).

In our recent study (Chaieb et al., 2009) blood oxygenation level dependent (BOLD) MRI was used to monitor modulations in human sensorimotor activity after the application of 4-min tRNS. This short-duration application of tRNS can induce a transient decrease in BOLD activity in the human primary sensorimotor cortex, using a classical finger-tapping task. If we

consider this 4-min stimulation effect as an inhibitory response, the result is at least at first glance, surprising. However, it is possible that different stimulation parameters can induce varying changes in the levels of cortical excitability. Another study using rTMS by Maeda et al. (2000) reported obtaining two varying responses with the same number of pulses in an rTMS paradigm: an increase in the amplitude of MEPs was observed after 1600 pulses of 10 Hz rTMS at 90% resting motor threshold, but the same effect was not observed by applying 1600 pulses at 1 Hz. According to the Bienenstock-Cooper-Munro (BCM) rule, a low overall cortical activity level is suggested to enhance the synaptic strength of active neuronal connections, while a consistently high level of activity should diminish it (Bienenstock et al., 1982). According to this rule, if we consider tRNS to be an excitatory stimulation, we should expect that a similarly excitability enhancing sensorimotor activity induces the inhibition that results in a decrease in BOLD response.

A previous study by Yamamoto et al. (2005) used a distinctly lower frequency range (< 2 Hz) in patients with Parkinson's disease. Their method, however, differed from ours in electrode position, stimulation amplitude, duration and techniques of evaluation. Improved autonomic and motor functions were detected after 24 hours of continuous noisy vestibular electrical stimulation over the bilateral mastoids. The authors hypothesized that in PD patients the input noise ameliorated the impaired neuronal transmission, with the noise enhancing the weak neuronal signal detection in the sensory system; a process known as stochastic resonance, and reported in several experimental studies (e.g. Moss et al., 2004).

Stochastic resonance may play a role in tRNS, however in a much higher frequency range. For some years now, oscillations in a frequency range of 80 to 200 Hz (ripples) have been associated with plasticity processes (Grenier et al., 2001) and learning (Ponomarenko et al., 2008). There is currently much research devoted to the role of neuronal synchrony in cognition and perception (for a review see Ward et al., 2006), explaining how a small amount of noise injected into a biological system can enhance the detectability of weak signals. If this is the case, then manipulations of neuronal oscillations can have far-reaching consequences in mechanisms of attentional processing and consciousness. A further mechanism of tRNS may be the activation of sodium channels via rectification by high frequency stimulation (Bromm, 1968). Recently it was shown that repetitive extracellular high-frequency stimulation in cultured rat neurones activated an inward sodium current which gives rise to a weak depolarization of the cell membrane (Schoen and Fromherz, 2008). Although the time integral of the stimulating current in the voltage clamp data study was zero, the average membrane potential was shifted in the direction of depolarization. This resulting depolarization was

claimed to be caused by the nonlinearity of the sodium current-voltage input during subthreshold excitation. Since we used a symmetric high frequency stimulation this non-linearity could be the reason for the excitatory effects we have seen with tRNS. Interestingly, the effect of tRNS increased with time after stimulation. Effects induced by "repetitive activation of Na^+ channels by weak capacitive currents" studied by Schoen and Fromherz (2008) also increase with stimulation time, however within a much shorter time range < 1s. On the other hand continuous opening of Na^+ channels would lead to membrane depolarization, from which we can assume from tDCS studies that a time range of > 3 minutes may lead to LTP- like mechanisms.

Thus, finally, the neuroplastic effects of tRNS could be related to anodal tDCS aftereffects, but with clear advantages. TRNS can circumvent problems which can arise by stimulating a folded cortex with anodal stimulation, since on one side of the gyrus wall, current orientation induces excitation, while on the opposite side of the gyrus, it will inevitably induce inhibition. When using tRNS only excitatory aftereffects are observable. Also "tangential" stimulation of nerve cells now appears to be possible with tRNS. Within a "tangential" DC electric field applied to a symmetrical dendritic arbour, currents on both sides would cancel each other at the axon hill. In the case of a rectifying depolarisation by fast oscillating field, the cell would be depolarised irrespective of current flow orientation. Safety concerns are probably lessened than in the case of tDCS. Several anecdotal, but so-far-unpublished, reports have described small skin burns after tDCS. In general, non-polarising currents seem to be safer than polarizing currents as seen in deep brain stimulation. Here we have not observed any tRNS induced changes with EEG recordings. TRNS using 1 mA was not noticeable by the subjects, compared with a slight skin tingling sensation associated with tDCS. Thus it appears to have the best blinding potential for controlled studies of presently available methods.

In summary, the transcranial application of weak AC current and random noise may appear to be a promising tool for clinical neuroplasticity research. They allow for a selective, focal, non-invasive and reversible excitability modulation of the cortex. Furthermore, tRNS allows an unnoticeable and thus painless way to induce increases in cortical excitability. The main advantage of tRNS seems to be the direction insensitivity characteristic of the stimulation. It seems to provide a qualitatively new way of producing and interfering with brain plasticity. However, important research still has to be done, mainly in uncovering the mode of action, and in finding a way to prolong the aftereffects of weak current application further, as has already successfully been done in DC research.

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Comparatively weak after-effects of transcranial alternating current stimulation (tACS) on cortical excitability in humans

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Objective
Interference with brain rhythms by noninvasive transcranial stimulation that uses weak transcranial alternating current may reveal itself to be a new tool for investigating cortical mechanisms currently unresolved. Here, we aim to extend transcranial direct current stimulation (tDCS) techniques to transcranial alternating current stimulation (tACS).

Background
Parameters such as electrode size and position were taken from those used in previous tDCS studies.

Methods
Motor evoked potentials (MEPs) revealed by transcranial magnetic stimulation (TMS), electroencephalogram (EEG)-power, and reaction times measured in a motor implicit learning task, were analyzed to detect changes in cortical excitability after 2-10 minutes of AC stimulation and sinusoidal DC stimulation (tSDCS) by using 1, 10, 15, 30, and 45 Hz and sham stimulation over the primary motor cortex in 50 healthy subjects (eight-16 subjects in each study).

Results
A significantly improved implicit motor learning was observed after 10 Hz AC stimulation only. No significant changes were observed in any of the analyzed frequency bands of EEG and with regard to the MEP amplitudes after AC or tSDCS stimulation. Similarly, if the anodal or cathodal DC stimulation was superimposed on 5, 10, and 15 Hz AC stimulation, the MEP amplitudes did not change significantly.

Conclusions
Transcranial application of weak AC current may appear to be a tool for basic and clinical research in diseases with altered EEG activity. However, its effect seems to be weaker than tDCS stimulation, at

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least in the present context of stimulus intensity and duration. Further studies are required to extend cautiously the safety range and uncover its influence on neuronal circuitries.
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Keywords transcranial alternating current stimulation; transcranial magnetic stimulation; electroencephalogram; motor cortex; serial reaction time task

Neuroplasticity is an ongoing, self-organizing, adapting process that is widespread in cortical areas; it allows the brain to learn and adapt to new environmental situations. External influences on neuroplastic processes may be used for functional improvement of diseases, in particular for improving cortical functions such as learning or for studying brain function per se. Several methods exist to influence excitability of the brain by external means. The most well-known is transcranial magnetic stimulation (TMS) introduced about 20 years ago.[1] It followed transcranial electrical pulsed stimulation, which because of its painful stimulation characteristic never proceeded to a routine application method.[2] Another electrical approach, weak direct current stimulation of the brain (transcranial direct current stimulation [tDCS]), was investigated intermittently within the last four decades, but entered into neurobiologic and clinical plasticity research[3,4] only after its efficacy was unambiguously demonstrated by quantifying its effects during and after stimulation by single-pulse TMS over the motor cortex.[5] TDCS is able to induce long-lasting changes in cortical excitability in different brain regions in a reversible, relatively selective, painless, and safe manner. Generally, motor cortex excitability is enhanced by anodal and decreased by cathodal stimulation, as seen in studies that used single-pulse TMS. Even though in humans the effects of tDCS were first demonstrated on the motor system, it also influences visual, somatosensory, and cognitive functions.[6,7]

Transcranial alternating current stimulation (tACS) of the brain is a new technique. It aims to interfere with ongoing oscillations in the brain. This technique may have important implications for neuropsychiatric disorders, for example, it has been concluded that measures of gamma synchrony offer a valuable window into the core integrative disturbance in schizophrenia.[8] Recently, it was shown that inducing slow oscillation-like potential fields by transcranial application of oscillating potentials (0.75 Hz) during early nocturnal nonrapid eye–movement sleep, (a period of emerging slow wave sleep) enhances the retention of hippocampus-dependent declarative memory in healthy humans.[9] The slowly oscillating potential stimulation induced an immediate increase in slow wave sleep, endogenous cortical slow oscillations, and slow spindle activity in the frontal cortex. Brain stimulation with oscillations at 5 Hz—another frequency band that normally predominates during rapid eye–movement sleep—decreased slow oscillations and left declarative memory unchanged.

The aim of the current study is to further expand the stimulation spectrum between DC and AC stimulation. For this, we defined a frequency spectrum between 1 and 45 Hz transcranial electrical stimulation and analyzed motor-evoked potentials (MEPs) and electroencephalogram (EEG) spectrum before and after AC stimulation, both with and without an anodal and cathodal DC shift. Intracellular and EEG recordings in animals[10] have shown that modulating the excitability of cortical pyramidal cells generates a powerful and coherent feedback to the thalamus, resulting in highly coherent oscillations similar to those measured during natural sleep. These experiments are compatible with a role of the cortex in triggering and synchronizing oscillations generated in the thalamus, through cortico-thalamico-cortico loops, thus providing a possible cellular mechanism to explain the origin of large-scale coherent oscillations in the thalamocortical system. By stimulating the sensorimotor cortex with the use of tACS, oscillations can be triggered and may also reset ongoing rhythmic activity of local pacemaker networks consequently synchronizing brain oscillations.

Furthermore, behavioral tasks were used to study AC-driven changes in performance during a variant of the serial reaction time task (SRTT),[11-13] which is a standard paradigm to test implicit motor learning. In this task, subjects perform finger movements repetitively without being aware of a sequential order. We applied tACS or sham stimulation to the primary motor cortex during performance of the task.

Methods and materials

Subjects

Fifty subjects (24 men and 26 women) participated in the studies. None of the subjects took regular or acute medication. Participants gave informed written consent. The experiments were approved by the Ethics Committee of the University of Göttingen, and conformed to the Declaration of Helsinki. All subjects were right handed, according to the Edinburgh handedness inventory.[14]

Transcranial alternating current stimulation (tACS)

Ten healthy subjects (22-43 years old, mean age = 26.4 ± 8.0, 3 men) participated in the TMS study. Eight healthy subjects (22-32 years old, mean age = 25.75 ± 3.28, 3

men) were involved in the EEG experiments. Two subjects participated in both the EEG and MEP experiments. Sixteen volunteers (22-31 years old, mean age = 22.4 ± 4.15, 7 men) took part in the implicit learning study.

Transcranial sinusoidal direct current stimulation (tSDCS)

Ten healthy subjects (23-30 years old, mean age = 28.7 ± 7.0, 6 men) were involved in the TMS study and 11 subjects took part in the EEG experiments (22-43 years old, mean age = 26.8 ± 5.7, 5 men).

tACS and tSDCS

Electrical stimulation was delivered by a battery-driven constant-current stimulator (NeuroConn GmbH, Ilmenau, Germany) through conductive-rubber electrodes, enclosed in two saline-soaked sponges. The stimulation electrode was placed over the left motor cortex, which was determined by single-pulse TMS. The reference electrode was placed over the contralateral orbit. The size of the stimulation electrode was 4 × 4 cm and the reference electrode was 5 × 10 cm. The electrodes were fixed by elastic bands. tACS was applied for 5 minutes with a current intensity of 400 μA and tSDCS for 2 or 4 minutes with a current intensity of 250 μA. Concerning tSDCS, the anodal or cathodal stimulation was sinusoidally modified. This kind of stimulation resulted in no change in polarity.

In the SRTT study, the current was delivered during the Blocks 2 to 5, which lasted approximately 7 minutes and in eight subjects during the Blocks 2 to 8, which lasted approximately 10 minutes. The current was always ramped up or down over the first and last 2 seconds of stimulation. The maximal current density was 25 $\mu A/cm^2$ in the case of tACS and 15.625 $\mu A/cm^2$ in the tSDCS experiments over the motor cortex, which is below the safety parameters accepted for tDCS.[15] The current density was 8 or 5 $\mu A/cm^2$ with regard to the reference electrode. For sham stimulation, the current was turned on for 8 seconds at the beginning of the stimulation to achieve the light itching sensation under the electrode. However, it was more difficult to establish a sham condition with tACS because the rapid changes in current amplitude and direction caused flickering at the higher frequencies (30 and 45 Hz) and experienced subjects might notice this sensation.

Subjects were blinded for stimulation conditions in all of the studies. In the case of tACS, the TMS study was double blind. However, as we mentioned previously, this statement with regard to the subjects was true only for the lower frequencies.

Transcranial magnetic stimulation (TMS)

TMS was performed by using a standard double ("figure-of-eight") 70-mm coil connected to monophasic Magstim200 stimulator (Magstim Company, Whiteland, Dyfed, UK). The coil was placed tangentially to the scalp, with the handle pointing posterolaterally at 45-degree angle from the midline. The optimum position was defined as the site where TMS resulted consistently in the largest MEP in the resting muscle. The site was marked with a waterproof skin marker to ensure that the coil was held in the correct position throughout the experimental sessions. Surface electromyography was recorded from the right first dorsal interosseus (FDI) muscle with the use of Ag-AgCl electrodes in a belly-tendon montage. The signals were amplified and filtered (with a low-pass filter of 3 kHz, sampling rate of 5 kHz), digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK), recorded by a computer that used SIGNAL software (Cambridge Electronic Design, version 2.13). Data were analyzed offline. Complete muscle relaxation was controlled online via auditory and visual feedback of electromyography activity.

RMT was defined as the lowest stimulus intensity, which elicited a peak-to-peak MEP-amplitude of 50 μV, or more in the resting muscle, in at least 3 of 6 recordings. AMT was the minimum intensity eliciting a MEP of a superior size compared with moderate spontaneous muscular background activity (approximately 15% of the maximum muscle strength) in at least 3 of 6 trials.[16] The intensity of the stimulator output for the single test-pulse MEP was adjusted so that TMS led to an average MEP amplitude of about 1 mV peak-to-peak before electrical stimulation. The same stimulus intensity was used after the tSDC and tACS.

EEG recording

The EEG was recorded by using a 3-channel montage. One electrode was placed over Oz and 2 laterally above the motor region (C3 and C4) in accordance with the international 10/20 system. The impedance was kept below 5 kΩ. Linked mastoids (RLm) were used as references, the ground electrode was positioned on the forehead. Data were collected with a sampling rate of 1000 Hz with the use of the BrainAmp system (Brain Products GmbH, Munich, Germany) and were analyzed offline (Brain Vision Analyzer, Brain Products GmbH).

Experimental design

Subjects were seated in a comfortable reclining chair with a mounted headrest throughout the experiments. Within each type of experimental technique, the MEP-EEG measurements were always performed by the same investigator.

TMS study

tACS

Ten subjects participated in 6 experimental sessions on separate days, 1 day apart to avoid carry over effects. The

TMS experiments were performed at identical times. The subjects received 1, 10, 15, 30, and 45 Hz tACS and sham stimulation in a randomized order. RMT, AMT, the intensity to evoke MEP of approximately 1 mV peak-to-peak amplitude, single-test pulse MEPs were recorded before tACS. Stimulus intensities (in percentage of maximal stimulator output) of TMS were determined at the beginning of each experiment. Thirty single-test pulse MEPs were recorded 7 times after the stimulation, ie, approximately 0 minutes after tACS, 2, 4, 7, 10, 15, and 20 minutes after the end of AC stimulation.

tSDCS

Ten subjects received anodal and 7 received cathodal tSDCS with a frequency 5, 10, and 15 Hz for 2 minutes in a counterbalanced order. Stimulations were performed on separate days and between each stimulation session was a 15-minute break. Fifty single-test pulse MEPs were recorded before and 40 MEPs after tSDCS (averaged in 20 blocks).

EEG study

tACS

The EEG experiments were conducted in a repeated measurement design using a randomized order, with a break of minimum 20 minutes between each stimulation session. A 2-minute EEG was recorded at rest before and 3 times after AC stimulation (immediately, 7, and 14 minutes after the end of the stimulation). Subjects received 1, 10, and 45 Hz tACS in a randomized and counterbalanced order.

tSDCS

The tSDCS was administered at 5, 10, and 15 Hz in a randomized order, with a 20-minute break between stimulation sessions. A 2-minute EEG was recorded before stimulation, and then a 4-minute EEG recorded immediately after stimulation. Subjects received tSDC for a 4-minute duration at an intensity of 250 μA in both an anodal and cathodal direction.

SRTT

Subjects were seated in front of a computer screen at eye level behind a response pad with 4 buttons numbered 1 to 4, and were instructed to push each button with a different finger of the right hand (index finger for button 1, middle finger for button 2, ring finger for button 3, and little finger for button 4). An asterisk appeared in 1 of 4 positions horizontally spaced on a computer screen and permanently marked by dots. The subjects were instructed to press the key corresponding to the position of the asterisk as fast as possible. After a button was pushed, the go signal disappeared. The next go signal was displayed 500 milliseconds later. The test consisted of 8 blocks of 120 trials. In Blocks 1 and 6, the sequence of asterisks followed a pseudorandom order in which asterisks were presented equally frequently in each position and never in the same position in 2 subsequent trials. In Blocks 2 to 5 and 7 and 8, the same 12-trial sequence of asterisk positions repeated itself 10 times (abadbcdacbdc). Subjects were not told about the repeating sequence.

Where improved performance during the whole course of the task is due to implicit learning as well as to increasing task routine, differences in performance between Block 5 and the random Block 6 represent a measure of implicit learning only, because task routine is thought to be equivalent in both blocks, and thus any differences in performance should be due to implicit sequence learning.[17]

In 6 subjects, the first 3 blocks of the previously used test was repeated 1 (Block 9: pseudorandom; Blocks 10 and 11 repeated sequences) and 2 hours (Block 12: pseudorandom; Blocks 13 and 14: repeated sequences) poststimulation. Differences in performance between Blocks 9 and 10 and 12 and 13 represent a measure of implicit learning.

Safety aspects

All the subjects completed a questionnaire the next day after the completion of the experimental sessions. The questionnaire contained rating scales for the presence and severity of headache, difficulties in concentrating, acute mood changes, visual perceptual changes, fatigue, and discomforting sensations like pain, tingling, itching, or burning under the electrodes during and after tACS.[18]

Analysis of the data

EEG analysis

EEG epochs (2 minutes) were segmented for 30 seconds and filtered by using 0.1 Hz (24 dB/octave) low cutoff and a 70 Hz (24 dB/octave) high cutoff and 50 Hz notch filters. In addition to semiautomatic artifact detection (200 μV amplitude criterion), all epochs were visually inspected, and those containing eye blinks or muscle movement artifacts were excluded. After artifact rejection all of the epochs were segmented into 2 seconds, and fast fourier transformation (FFT) was calculated for all electrodes (0.5 Hz resolution, and 10% Hamming-window). The FFT segments were averaged for each 30 seconds. The mean activity in voltage was calculated and exported from each frequency bands (theta band 4.5-7 Hz, alpha band 8-12 Hz, beta band 12.5-30 Hz, and gamma band 31-49 Hz) for statistical analysis.

MEP analysis

Peak-to-peak amplitudes (mV) of each MEP were measured offline, and mean MEP amplitudes were calculated for each stimulation condition for each time point separately. The amplitudes were normalized to baseline.

SRTT analysis

In each trial, response time (RT) was measured from the appearance of the go signal until the first button was pushed by the subject. For each block of trials of a given experimental condition, mean RT was calculated for each subject separately. Furthermore, the SD of RTs for each subject in every block was calculated as an index of variability of RTs. An error rate (ER) was calculated to assess the number of incorrect responses for each block and each subject in each stimulation condition.

Statistical analysis

In all of the EEG and TMS experiments a repeated measures of analysis of variance (ANOVA) (a given current condition versus sham $\times$ time points of poststimulation; dependent variable: mean amplitude of MEPs, FFT power in a given frequency band) was calculated. In case of the significant interaction of time and stimulation condition, a post hoc test was performed.

Concerning the implicit learning statistical analyses were performed with repeated measures of ANOVA (current conditions $\times$ 8 blocks) for RT, ER, and variability. Because the RT and ER differences between Blocks 5 and 6 are thought to represent an exclusive measure of implicit learning, interactive Student t tests[19] were performed to compare the respective differences for the alternating current stimulation condition versus sham condition.

Results

All the subjects tolerated the stimulation; none of the experimental sessions were interrupted because of side effects of the stimulation. However, about half of the subjects noticed light flickering during higher frequency stimulation (30, 45 Hz) by using an intensity of 0.4 mA. As a result, we did not further increase the stimulation amplitude for safety reasons. Only 2 of the subjects reported a light burning sensation under the electrodes during the stimulation. Six subjects experienced a mild headache that lasted for a maximum of 2 hours after the end of tACS, independent from the frequency of the stimulation. None of the subjects reported transient side effect according to the 1 to 2 weeks' follow-up.

MEP

tACS

Repeated measurements of ANOVA (1, 10, 15, 30, 45 Hz versus sham stimulation condition separately $\times$ 7 time points poststimulation) revealed no significant interactions between current condition and time in any of the comparisons between tACS and sham stimulation. For 1-Hz stimulation, there was no main effect of stimulation [$F(1,18) = 0.03$, $P = .86$] and time course [$F(6,108) = 1.49$, $P = .19$]. The interaction between stimulation und time was also not significant [$F(6,108) = 1.17$, $P = .32$]. For 10-Hz stimulation, there was no main effect of stimulation [$F(1,18) = 3.2$, $P = .09$] and time course [$F(6,108) = 1.0$, $P = .42$]. The interaction between stimulation and time was also not significant [$F(6,108) = 0.61$, $P = .72$]. For 15-Hz stimulation, there was no main effect of stimulation [$F(1,18) = 0.004$, $P = .95$] and time course [$F(6,108) = 0.62$, $P = .71$]. The interaction between stimulation and time was also not significant [$F(6,108) = 1.77$, $P = .11$]. For 30-Hz stimulation, there was no main effect of stimulation [$F(1,18) = 0.14$, $P = .71$] and time course [$F(6,108) = 0.05$, $P = .99$]. The interaction between stimulation and time was also not significant [$F(6,108) = 1.03$, $P = .4$]. For 45-Hz stimulation, there was no main effect of stimulation [$F(1,18) = 0.01$, $P = .91$] and time course [$F(6,108) = 0.71$, $P = .64$]. The interaction between stimulation and time was also not significant [$F(6,108) = 0.4$, $P = .87$]. Table 1 shows the mean MEP values and their standard error before and after tACS.

tSDCS

Here, anodal or cathodal stimulation was sinusoidally modified at a given frequency. The ANOVA was calculated separately for anodal and cathodal stimulation (5-, 10-, and 15-Hz stimulation conditions and 2 time points poststimulation). The analysis revealed no significant interactions between current condition and time in either the anodal or cathodal condition ($F < 1.2$, $P > .3$). An increase in motor-cortical excitability, after the combination of anodal and 15-Hz stimulation, of approximately 35% was observed after stimulation, but was not significant when compared with the baseline values ($P = .08$). Table 2 shows the mean MEP values and their standard error before and after tSDCS.

Table 1 Mean MEP amplitudes (SEM) before and after tACS at 1-, 5-, 10-, 15-, and 30-Hz stimulation

	1 Hz	10 Hz	15 Hz	30 Hz	45 Hz	Sham
Before	1.02 ± 0.11	1.03 ± 0.13	1.03 ± 0.09	1.03 ± 0.08	1.04 ± 0.09	1.02 ± 0.11
0 min	1.01 ± 0.30	0.93 ± 0.31	1.15 ± 0.37	1.06 ± 0.33	1.15 ± 0.46	1.19 ± 0.42
2 min	1.04 ± 0.44	0.94 ± 0.31	1.05 ± 0.41	1.11 ± 0.38	1.11 ± 0.47	1.20 ± 0.38
4 min	1.16 ± 0.37	0.91 ± 0.37	1.17 ± 0.34	1.16 ± 0.33	1.30 ± 0.51	1.20 ± 0.31
8 min	1.14 ± 0.35	0.92 ± 0.43	0.98 ± 0.27	1.15 ± 0.29	1.19 ± 0.45	1.20 ± 0.36
10 min	1.20 ± 0.45	0.99 ± 0.36	1.13 ± 0.37	1.14 ± 0.29	1.06 ± 0.51	1.31 ± 0.46
15 min	1.32 ± 0.53	1.08 ± 0.40	1.13 ± 0.27	1.20 ± 0.20	1.09 ± 0.41	1.16 ± 0.41
20 min	1.27 ± 0.52	0.99 ± 0.27	1.21 ± 0.20	1.11 ± 0.33	1.06 ± 0.43	1.04 ± 0.22

A decrease of the MEP amplitude after 10-Hz stimulation was observed, but was not significant.

Table 2 Mean MEP amplitudes (SEM) before and after tSDCS at 5-, 10- and 15-Hz stimulation

	Anodal (mean MEPs and SEM)			Cathodal (mean MEPs and SEM)		
	Before	2 min after	4 min after	Before	2 min after	4 min after
5 Hz	1.12 ± 0.1	1.12 ± 0.2	1.16 ± 0.2	0.92 ± 0.06	1.08 ± 0.14	0.8 ± 0.15
10 Hz	1.04 ± 0.08	1.27 ± 0.1	1.13 ± 0.1	0.97 ± 0.06	0.92 ± 0.11	0.94 ± 0.16
15 Hz	1.2 ± 0.03	1.6 ± 0.2	1.37 ± 0.11	0.89 ± 0.1	1.08 ± 0.2	0.9 ± 0.2

An increase of the MEP amplitude after anodal 15 Hz stimulation was observed, but was not significant.

EEG

tACS

The ANOVA (1-, 10-, and 45-Hz stimulation conditions × 16 time points – including baseline × 3 channels) was calculated separately for each frequency band. It revealed no significant interactions among current conditions, time, and channels at any of the different frequencies applied. The results of the statistical analysis are summarized in Table 3.

tSDCS

The ANOVA was calculated for anodal and cathodal directions and for each frequency band separately. The analyses (5-, 10-, and 15-Hz stimulation conditions × 16 time points – including baseline × 3 channels) revealed no significant interactions between current conditions and time at any of the different frequencies applied ($F < 0.5$, $P > .4$). The results of the statistical analysis are summarized in Table 4.

SRTT

RTs of the SRTT shortened during tACS of the primary motor cortex; repeated measures ANOVA (1-, 10-, 45-Hz, and sham stimulation conditions × 8 blocks) revealed a significant effect on blocks [$F(7,105) = 33.11$; $P < .001$]. This was caused by an interaction of alternating current versus sham stimulation for Block 5 and Block 6, caused by a greater difference in the alternating current stimulation in the case of 10-Hz stimulation ($t = -3.26$, $df = 15$, $P = .005$) as revealed by Student t tests. Figure 1 shows the differences between 10-Hz and sham stimulation. Despite the significant main effect of blocks in ANOVA, the results of all other tests remained insignificant. However, a trend toward reduced RTs in Blocks 2 to 5 and 7 and 8 for tACS compared with the sham condition was identified.

The paradigm was repeated in 6 subjects after 1 and 2 hours poststimulation. At these time points the RTs were not different between the 10-Hz and sham stimulation conditions (Fig 1).

To exclude the possibility that tACS speeds up the initiation of movements and does not modify implicit learning, the 10-Hz and sham stimulations were repeated during Block 2 to 8 in 8 subjects by using different sequences than before. During the random block (Block 6) tACS and sham stimulation did not have a differential effect on RTs ($P = .64$). However, the ratio of Block 5 and Block 6, because of a greater difference in the 10-Hz stimulation condition remained significant ($t = -2.41$, $df = 7$, $P = .046$).

For ER, the ANOVAs showed a significant main effect on current ($P < .001$) and blocks at 1 Hz ($P = .012$) and at 45 Hz ($P = .001$), there was no significant condition × blocks interaction. Student t tests revealed no significant

Table 3 The results of the repeated measures ANOVA of the EEG experiment with tACS

	df	F value	*P* value
Theta band			
Stim	2	0.037	.964
Channel	2	82.709	<.001*
Time	15	1.843	.028*
Stim × Channel	4	0.130	.971
Stim × Time	30	1.021	.440
Channel × Time	30	1.010	.453
Stim × Channel × Time	60	1.198	.154
Alpha band			
Stim	2	0.002	.998
Channel	2	13.614	<.001*
Time	15	6.343	<.001*
Stim × Channel	4	0.053	.995
Stim × Time	30	0.674	.904
Channel × Time	30	1.125	.298
Stim × Channel × Time	60	0.837	.803
Beta band			
Stim	2	0.089	.916
Channel	2	14.952	<.001*
Time	15	1.102	.353
Stim × Channel	4	0.054	.994
Stim × Time	30	0.774	.799
Channel × Time	30	1.017	.442
Stim × Channel × Time	60	0.648	.981
Gamma band			
Stim	2	0.106	.900
Channel	2	0.097	.908
Time	15	0.723	.761
Stim × Channel	4	0.193	.941
Stim × Time	30	0.572	.967
Channel × Time	30	1.590	.025*
Stim × Channel × Time	60	0.771	.896

Independent variables: time course (Time), condition of current stimulation (Stim), and EEG channels (Channel); dependent variable: FFT power in a given frequency band. The ANOVA revealed no significant interactions between current conditions, time, and channels at any of the different frequencies applied. The asterisk indicates significant *P*-values ($P < .05$).

Table 4 The results of the repeated measures ANOVA of the EEG experiment with tSDCS

	Anodal			Cathodal		
	df	F value	*P* value	*df*	F value	*P* value
Theta band						
Stim	2	1.085	.351	2	0.194	.824
Channel	2	0.511	.602	2	3.149	.05*
Time	7	1.191	.309	7	2.821	.007*
Stim × Channel	4	0.901	.469	4	0.706	.591
Stim × Time	14	0.469	.947	14	1.121	.34
Channel × Time	14	0.857	.606	14	0.92	.536
Stim × Channel × Time	28	1.143	.283	28	1.157	.268
Alpha band						
Stim	2	0.011	.989	2	0.861	.433
Channel	2	1.026	.365	2	0.926	.401
Time	7	1.062	.388	7	1.734	.102
Stim × Channel	4	0.492	.742	4	0.952	.44
Stim × Time	14	0.464	.949	14	0.717	.755
Channel × Time	14	1.456	.124	14	0.579	.881
Stim × Channel × Time	28	1.169	.255	28	1.348	.113
Beta band						
Stim	2	0.092	.912	2	1.076	.353
Channel	2	2.292	.109	2	1.784	.176
Time	7	2.94	.005	7	0.713	.66
Stim × Channel	4	0.987	.422	4	0.201	.936
Stim × Time	14	0.404	.972	14	0.888	.572
Channel × Time	14	1.016	.436	14	1.426	.136
Stim × Channel × Time	28	0.901	.614	28	1.038	.413
Gamma band						
Stim	2	0.135	.874	2	0.51	.605
Channel	2	0.258	.772	2	1.559	.218
Time	7	2.286	.028	7	0.735	.641
Stim × Channel	4	0.444	.776	4	0.37	.828
Stim × Time	14	0.377	.98	14	0.806	.661
Channel × Time	14	0.659	.813	14	3.19	.001*
Stim × Channel × Time	28	0.762	.804	28	0.689	.884

Independent variables: time course (Time), condition of current stimulation (Stim) and EEG channels (Channel); dependent variable: FFT power in a given frequency band. The ANOVA revealed no significant interactions between current conditions, time, and channels at any of the different frequencies applied. The asterisk indicates significant *P*-values ($P < .05$).

difference between Block 5 and Block 6. For variability, the ANOVAs showed a significant main effect of condition ($P < .001$) and blocks ($P < .001$) without significant interaction between condition and blocks at all frequencies. Student *t* tests revealed no significant difference between Blocks 5 and 6.

Comment

The main result of the current study is that 10-Hz tACS over the motor cortex by using 7-minute stimulation duration was able to improve implicit motor learning. However, 10 Hz AC stimulation did not modify the EEG power and the MEP amplitudes significantly, when compared with sham stimulation.

In our study, only 10 Hz of tACS significantly improved performance in the acquisition and early consolidation phase of implicit motor learning. Compared with the noncurrent stimulation condition, reaction times in the SRTT decreased significantly and became faster during the course of the experiment. This result is similar to the effect of anodal stimulation over the M1 reported by a previous study.[20] Previous studies suggest that an excitability enhancement seems to be a necessary condition for learning by inducing strengthening of synapses/long-term-potentiation by modifying NMDA-receptor efficacy.[21,22] Regarding studies in the human, this is in line with observations of increased activation of the motor cortex during motor learning tasks,[23,24] and also with pharmacologic results showing that results of motor training can be improved by cortical excitability enhancements.[25] It appears that a 10-Hz tACS-driven cortical excitability change could facilitate the learning process.

The nonsignificant MEP changes were probably caused by the low number of subjects, larger sample sizes would have been necessary to prove the significant effect of

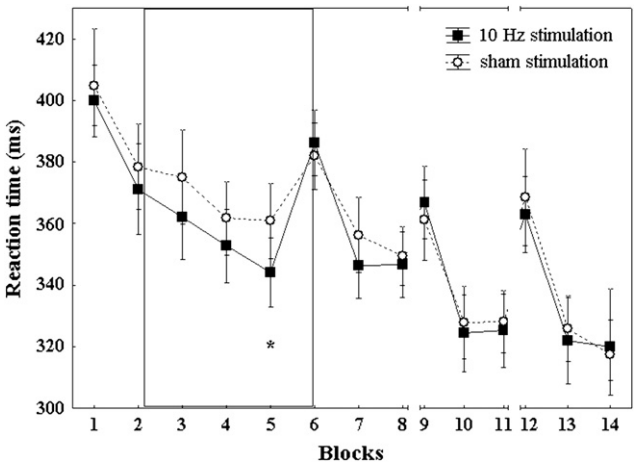

Figure 1 10-Hz tACS of the primary motor cortex improves implicit motor learning in its early phase. Reaction times decrease faster in the 10 Hz stimulation condition when compared with the sham-stimulation condition. Moreover, the RT difference comparing Blocks 5 and 6, which indicates implicit sequence learning, is larger for the 10-Hz stimulation condition, when compared with the nonstimulation condition. The asterisk indicates a significant difference regarding reaction time differences between Blocks 5 and 6, and between 10-Hz and sham stimulation. In 1 and2 hours poststimulation, this significant difference was not detectable any more.

stimulation if there is any. However, we also noticed that the SDs of the MEP amplitudes were higher when compared with those measured by previous studies that used tDCS.[3,4] Similar to our results, it was also published that there was no significant change in corticospinal excitability after 10-Hz rTMS.[26] Nevetheless, in our study 10 Hz of tACS had a facilitatory effect on motor performance. The only difference between the 2 tACS studies was the duration of the stimulation: in the TMS study, a shorter stimulation duration was applied than that used in the implicit learning study. Therefore, it may be possible that the effect of 10 Hz tACS is stimulation duration-dependent: a shorter stimulation duration may not be strong enough to modify cortical excitability, whereas a longer stimulation duration may have facilitatory effects.

We used a relatively small stimulation electrode size in order to enhance the focality of stimulation[27] and larger reference electrode to avoid the stimulation of the frontopolar cortex and retina. However, half of the subjects still noticed a flickering in their visual field, mainly during high-frequency stimulation. This means that at 30 and 45 Hz stimulation conditions in our study was not precisely "double-blind" per se because the subjects could feel the difference between sham and verum stimulation. This problem with sham stimulation was not encountered with tDCS.[18] Further increase of the reference electrode size might help but technically is not possible therefore, in the future, systematically exploring the effect of electrical stimulation with the use of new electrode positions (eg, M1–occipital cortex or extracephalic reference) is necessary. If intensities are comparable between tDCS and tACS 0.4 mA might be at the lower border for inducing after effects.[3] Thus, it remains to be seen if higher intensities or longer stimulation durations are better for inducing after effects, notwithstanding the assumption that higher stimulation intensities could be potentially more dangerous with respect to seizure induction. Along this suspicion, a similar new method, called focal electrically administered therapy (FEAT), uses also AC current. FEAT can be boosted to induce focal seizures, then referred to as "focal electrically administered seizure therapy" (FEAST).[28] This form of AC stimulation is intended to be used for a more focal form of electroconvulsive therapy in the future.

In conclusion, the transcranial application of weak AC current may appear to be a promising tool for clinical neuroplasticity research, for it allows a painless, selective, focal, noninvasive, and reversible excitability modulation of the cortex. Important research still has to be performed, mainly in uncovering the mode of function and in finding a way to prolong the effects of weak current application further, as has already been successfully done in DC research.

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Development/Plasticity/Repair

Increasing Human Brain Excitability by Transcranial High-Frequency Random Noise Stimulation

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For >20 years, noninvasive transcranial stimulation techniques like repetitive transcranial magnetic stimulation (rTMS) and direct current stimulation (tDCS) have been used to induce neuroplastic-like effects in the human cortex, leading to the activity-dependent modification of synaptic transmission. Here, we introduce a novel method of electrical stimulation: transcranial random noise stimulation (tRNS), whereby a random electrical oscillation spectrum is applied over the motor cortex. tRNS induces consistent excitability increases lasting 60 min after stimulation. These effects have been observed in 80 subjects through both physiological measures and behavioral tasks. Higher frequencies (100–640 Hz) appear to be responsible for generating this excitability increase, an effect that may be attributed to the repeated opening of Na^+ channels. In terms of efficacy tRNS appears to possess at least the same therapeutic potential as rTMS/tDCS in diseases such as depression, while furthermore avoiding the constraint of current flow direction sensitivity characteristic of tDCS.

Key words: **transcranial random noise stimulation (tRNS); primary motor cortex (M1); transcranial magnetic stimulation (TMS); serial reaction time task (SRTT); human; neuromodulation**

Introduction

Neuroplasticity is an ongoing, self-organizing, adaptive process widespread in cortical areas; it allows the brain to learn and adapt to new environmental situations. External influences on neuroplastic processes may be used for functional improvement of diseases, in particular for improving cortical functions such as learning. The most well known method currently used to influence excitability of the brain by external means is transcranial magnetic stimulation (TMS) (Barker et al., 1985). It was followed by various repetitive stimulation paradigms, most recently by theta burst stimulation (TBS) (Huang et al., 2005). Although TBS increased the efficacy of rTMS by reducing stimulus intensity and the number of pulses required to achieve similar aftereffects, its upper safety limits are still unclear due to the potential risk of rTMS inducing seizures (Wassermann, 1998).

Another approach, weak transcranial direct current stimulation (tDCS) of the brain has so far avoided this risk. tDCS was investigated intermittently within the last four decades, but entered into neurobiological and clinical plasticity research only after its efficacy for modulating neuroplasticity could be unambiguously quantified by comparing TMS-induced motor-evoked potentials (MEPs) before and after tDCS (Nitsche and Paulus, 2000, 2001). When compared with pulsed rTMS, tDCS represents the other end of the stimulation spectrum by delivering continuous electric current, which leads to "brain polarization." tDCS is able to induce long-lasting changes in cortical excitability in a reversible, relatively selective, painless, and safe manner. Generally, motor cortex (M1) excitability is enhanced by anodal and decreased by cathodal stimulation (Nitsche and Paulus, 2000).

Transcranial random noise stimulation (tRNS) of the human brain is a new technique (Fig. 1). Only one study so far has used noisy galvanic stimulation at a very low-frequency (<2 Hz) range targeting the vestibular nerves of patients with levodopa-responsive and unresponsive parkinsonism over a 24 h period (Yamamoto et al., 2005) and successfully improving parkinsonian symptoms. In this article, we demonstrate a new method of enhancing corticospinal excitability as measured by TMS, by applying weak tRNS for 10 min over the M1. Furthermore, a behavioral task was used to study tRNS-driven changes in performance during a variant of the serial reaction time task (SRTT) (Nissen and Bullemer, 1987), which is a standard paradigm to test implicit motor learning. In addition, we show how a cognitive or motor activity performed during stimulation can reduce the efficacy of tRNS, as previously described in studies using tDCS (Antal et al., 2007). The repeated potentiation of sodium channels has been suggested to be a putative mechanism of tRNS action; its aftereffects may outlast those observed after tDCS stimulation.

Materials and Methods

Subjects

Altogether, 80 healthy volunteers (32 men and 48 women; mean age, 25.74 ± 5.13 years; age range, 20–44 years) were informed about all aspects of the experiments, and all gave informed consent. None of the subjects suffered from any neurological or psychological disorders, had metallic implants/implanted electric devices, or took any medication regularly, and none of them took any medication in the 2 weeks before their

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The authors declare no competing financial interests.

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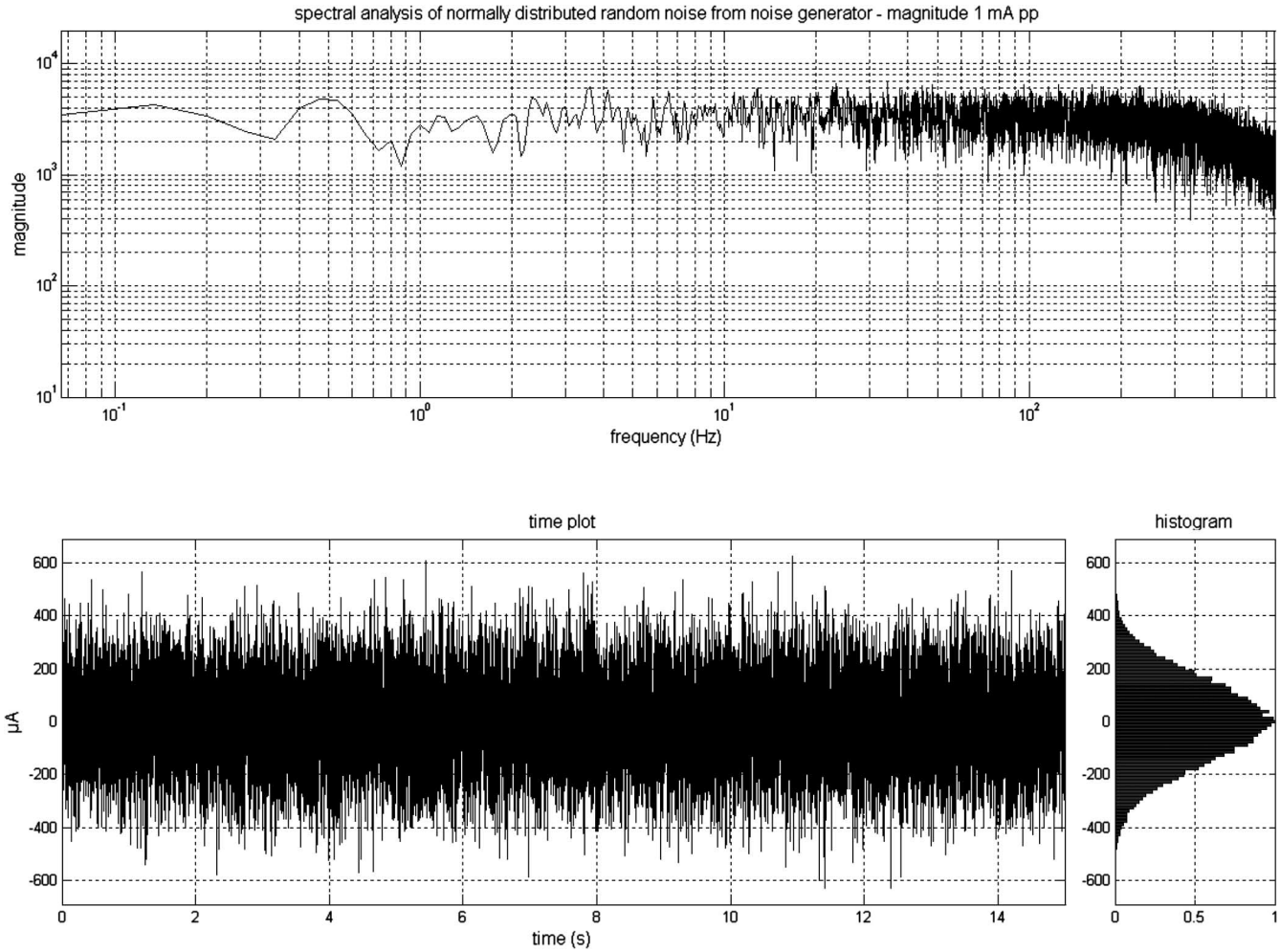

Figure 1. The output signal of DC-Stimulator PLUS, as a frequency distribution of the signal, the time plot of the signal, and a histogram. The signal was generated by a computer. In the stimulation mode "noise," there is a random level of current generated for every sample (sampling rate 1280 samples/s). The random numbers are normally distributed; the probability density function follows a bell-shaped curve. The amplitude of 1 mA pp means that 99% of all generated amplitude values were between +500 μA and −500 μA.

participation in any of the experiments. All subjects were right handed, according to the Edinburgh handedness inventory (Oldfield, 1971). We conformed to the Declaration of Helsinki, and the experimental protocol was approved by the Ethics Committee of the University of Göttingen.

Altogether, 47 healthy subjects (motor cortex: 17 participants; 21–27 years old; mean age = 23.71 ± 2.08; 6 male; low-frequency/high-frequency: 12 participants; 20–28 years old; mean age = 23.83 ± 3.28; 7 male; DC-shift-induced excitability changes: 8 participants; 22–38 years old; mean age = 25 ± 5.12; 4 male; premotor cortex: 10 subjects; 22–39 years old; mean age = 26.5 ± 6.31; 4 male) participated in the single-pulse TMS study. Ten healthy subjects (22–44 years old; mean age = 27.6 ± 6.67; 3 male) were involved in the paired-pulse TMS experiments, and four subjects participated in both single- and paired-pulse MEP experiments. Seventeen volunteers (22–31 years old; mean age = 25.29 ± 2.89; 8 male) took part in the implicit learning study. Twelve subjects were involved in the task-related modulation study (22–44 years old; mean age = 26.75 ± 6.08; 4 male). Three subjects participated both in the single-pulse MEP and in the implicit learning experiment. Two subjects were involved in both the single-pulse MEP and task-related modulation experiment.

Random noise stimulation

Stimulation was delivered by a battery-driven electrical stimulator (Version eldith DC-Stimulator-Plus, neuroConn) through conductive-rubber electrodes, placed in two saline-soaked sponges. In the stimulation mode "noise" there is a random level of current generated for every sample (sampling rate 1280 samples/s). The random numbers are normally distributed; the probability density function follows a bell-shaped curve. In the frequency spectrum all coefficients have a similar size ("white noise"). The noise signal contains all frequencies up to half of the sampling rate, i.e., a maximum of 640 Hz (Fig. 1). In a second experiment this frequency spectrum was separated into a low (0.1–100 Hz)- and high (101–640 Hz)-frequency spectrum. Because of the statistical characteristics, the signal has no DC offset, provided that the offset is set to zero.

The stimulation electrode was placed over the left motor cortex, which was determined by single pulse TMS. During the premotor single-pulse TMS study, the stimulation electrode was placed over the premotor cortex (2.5 cm anterior from the motor cortex). To identify the primary motor and premotor cortex the same method was used as that implemented in previous TMS and tDCS studies (e.g., Fink et al., 1997; Münchau et al., 2002). The reference electrode was placed over the contralateral orbit. The size of the stimulation electrode was 4 × 4 cm and the reference electrode was 6 × 14 cm. The electrodes were fixed by elastic bands. tRNS was applied for 10 min with a current strength of 1000 μA. The maximal current density was 62.5 $\mu A/cm^2$ over the motor cortex, which is below the safety parameters accepted for tDCS (Nitsche et al., 2003). The current density was 11.9 $\mu A/cm^2$ at the reference electrode. For sham stimulation the current was applied for 30 s at the beginning of the stimulation session, and then turned down. However, the screen on the stimulator did show the remaining time until the end of the stimulation session, as per the verum stimulation condition. Subjects were blinded for stimulation conditions in all of the studies.

Electrophysiological studies: transcranial magnetic stimulation

To detect current-driven changes of excitability, motor-evoked potentials (MEPs) of the right first dorsal interosseus muscle (FDI) were recorded following stimulation of its motor-cortical representation field by single-pulse TMS. These were induced using a Magstim 200 magnetic stimulator, with a figure-of-eight standard double magnetic coil (diameter of one winding, 70 mm; peak magnetic field, 2.2 T; average inductance, 16.35 μH). The coil was connected to two monophasic Magstim 200 stimulators via a bistim module during the paired-pulse TMS study. Surface electromyogram (EMG) was recorded from the right FDI through a pair of Ag–AgCl surface electrodes in a belly–tendon montage. Raw signals were amplified, bandpass filtered (2 Hz to 3 kHz; sampling rate, 5 kHz), digitized with a micro 1401 AD converter (Cambridge Electronic Design) controlled by Signal Software (Cambridge Electronic Design, version 2.13), and stored on a personal computer for off-line analysis. Complete relaxation was controlled through auditory and visual feedback of EMG activity and whenever it was necessary, the subject was instructed to relax. The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline, resulting in a posterior–anterior direction of current flow in the brain. This orientation of the induced electrical field is thought to be optimal for predominantly transsynaptic mode of activation of corticospinal system. The optimum position was defined as the site where TMS resulted consistently in the largest MEP in the resting muscle. The site was marked with a skin marker to ensure that the coil was held in the correct position throughout the experiment.

Experimental design

Subjects were seated in a comfortable reclining chair with a mounted headrest throughout the experiments. Within each type of experimental session, the measurements were always performed by the same investigator.

Single-pulse TMS

Motor cortex stimulation. Seventeen subjects participated in two experimental sessions, on separate days, at least 3 d apart to avoid carryover effects. The subjects received RN and sham stimulation in a randomized order. Resting motor threshold (RMT), active motor threshold (AMT), the intensity to evoke MEP of ~1 mV peak-to-peak amplitude (SI1mV), and a baseline of TMS-evoked MEPs (40 stimuli) were recorded at 0.25 Hz before the stimulation.

Stimulus intensities (in percentage of maximal stimulator output) of TMS were determined at the beginning of each experiment. RMT was defined as the minimal output of the stimulator that induced a reliable MEP (~50 μV in amplitude) in at least three of six consecutive trials when the FDI muscle was completely relaxed. AMT was defined as the lowest stimulus intensity at which three of six consecutive stimuli elicited reliable MEP (~200 μV in amplitude) in the tonically contracting FDI muscle (Rothwell et al., 1999).

Following stimulation, 40 single test-pulse MEPs were recorded at 0.25 Hz, i.e., ~0, 5, and 10 min after stimulation and then every 10 min up to 60 min.

Additionally, eight subjects underwent the same single-pulse TMS experiment (as described previously) to investigate the length of the aftereffect of the stimulation. Subjects were measured 0, 5, and 10 min after stimulation, then every 10 min up to 60 min, then twice in the second hour, then 4, 6, and 24 h after stimulation. Both active and sham stimulation conditions were applied.

In a second sham-controlled experiment, the random noise frequency was divided into a low (0.1–100 Hz)- and high (101–640 Hz)-frequency spectrum. Twelve participants underwent the same protocol as previously described.

To conclusively exclude DC-shift-induced excitability changes, eight subjects underwent the same protocol as previously described, in which the standard DC electrode montage was used (active electrode, anodal; reference electrode, cathodal) and then the electrode montage was reversed (cathodal–anodal).

Premotor cortex stimulation. Ten subjects participated in two experimental sessions on separate days, at least 3 d apart to avoid carryover effects. The subjects received tRNS and sham stimulation in a randomized order. The study protocol was performed as previously described.

Paired-pulse TMS

TMS measurements included RMT, AMT, and SI1mV, short-interval intracortical inhibition (SICI)/intracortical facilitation (ICF), long-interval intracortical inhibition (LICI), recruitment curves, and cortical silent period (CSP).

Ten subjects participated in four experimental sessions [(1) tRNS: recruitment curves and SICI/ICF; (2) tRNS: LICI and CSP; (3) sham: recruitment curves and SICI/ICF; and (4) sham: LICI and CSP] on separate days at least 3 d apart to avoid carryover effects. The subjects received RN and sham stimulation in a randomized order. Stimulus intensities (in percentage of maximal stimulator output) of TMS were determined at the beginning of each experiment. SI1mV was determined with single-pulse TMS first (the amplitude of the test MEP was matched before and after tRNS). RMT and AMT were defined as previously mentioned.

SICI/ICF and LICI were measured with two different protocols of single- and paired-pulse TMS applied in a random order at 0.25 Hz. For SICI/ICF, two magnetic stimuli were given through the same stimulating coil, and the effect of the first (conditioning) stimulus on the second (test) stimulus was investigated (Kujirai et al., 1993). To avoid any floor or ceiling effect, the intensity of the conditioning stimulus was set to a relatively low value of 80% of AMT. The test-stimulus intensity was adjusted to SI1mV. SICI was measured with interstimulus intervals (ISI) of 2 and 4 ms, and ICF with ISIs of 9, 12, 15, and 25 ms. The control condition (test pulse alone) was tested 40 times, and each of the conditioning-test stimuli 20 times. The mean peak-to-peak amplitude of the conditioned MEP at each ISI was expressed as a percentage of the mean peak-to-peak size of the unconditioned test pulse. The second protocol tested LICI with two suprathreshold stimuli applied with ISIs of 50, 100, 150, and 200 ms (Valls-Solé et al., 1992). The intensity of both stimuli was set to 110% of RMT. Here as well, the intensity was set to this relatively low value to avoid any floor or ceiling effect. The control condition (first pulse alone) was tested 40 times, whereas each of the paired stimuli was tested 20 times. LICI was taken as the mean percentage inhibition of conditioned MEP at ISIs of 50, 100, 150, and 200 ms.

Recruitment curves were measured with three different and increasing stimulus intensities (110%, 130%, and 150% of RMT), each with 10 pulses. A mean was calculated for all intensities. Finally, 10 pulses with SI1mV and 10 pulses with 120% RMT were applied under tonic contraction of the right first dorsal interosseus muscle. CSPs were separately determined, in rectified and averaged EMG traces with a prestimulus period of 100 ms. CSP (in ms) was measured from the TMS stimulus to the point where the signal reached the amplitude of the mean prestimulus EMG activity again for >5 ms.

Behaviorial studies

SRTT

Subjects were seated in front of a computer screen at eye level behind a response pad with four buttons numbered 1–4 and were instructed to push each button with a different finger of the right hand (index finger for button 1, middle finger for button 2, ring finger for button 3, and little finger for button 4). An asterisk appeared in one of four positions that were horizontally spaced on a computer screen and permanently marked by dots. The subjects were instructed to press the key corresponding to the position of the asterisk as fast as possible. After a button was pushed, the go signal disappeared. The next go signal was displayed 500 ms later. The test consisted of eight blocks of 120 trials. In blocks 1 and 6, the sequence of asterisks followed a pseudorandom order in that asterisks were presented equally frequently in each position and never in the same position in two subsequent trials. In blocks 2–5, 7, and 8, the same 12-trial sequence of asterisk positions repeated itself 10 times (abadbcdacbdc). Subjects were not informed about the repeating sequence.

In six subjects, the first three blocks of the previously used test were repeated 1 (block 9: pseudorandom; blocks 10–11: repeated sequences) and 2 h (block 12: pseudorandom; blocks 13–14: repeated sequences)

after stimulation. Differences in performance between blocks 9–10 and 12–13 also represent a measure of implicit learning. In the SRTT study, the current was delivered during the blocks 2–5, which lasted ~7 min. The order of verum and sham stimulation was randomized. The current was always ramped up or down over the first and last 2 s of stimulation.

Task-related modulation of tRNS

The three experimental sessions were conducted in a repeated-measurement design using a randomized order, with a break of at least 3 d between each session. First, the left motor-cortical representational field of the right FDI was identified using TMS. After determining the resting and active motor thresholds, a baseline of TMS-evoked MEPs (25 stimuli) was recorded at 0.25 Hz. Afterward, one stimulation electrode was fixed at the representational field of the right FDI, and the other was fixed at the contralateral forehead above the orbita.

During tRNS, subjects were passively sitting during the stimulation (experiment 1), had their attention directed toward a cognitive test (experiment 2) or were instructed to push a ball in their right hand (experiment 3). After termination of RNS, 25 MEPs were recorded every fifth minute up to 30 min and then every 15 min up to 2 h.

During the stimulation in experiment 2, the subjects were required to fill out a cognitive test that was presented on a computer monitor. The subjects had to push a suitable button with their right index finger to give the correct answer. The test was presented in German and downloaded from a commercial intelligence test homepage. The questions were on a variety of subjects. In experiment 3, the subjects were instructed to push a ball (8 cm diameter) in their right hand. The ball was connected to a display where the actual values related to pressure were quantified. Before the stimulation session, the subjects were asked to push the ball as hard as possible. During the tRNS session, subjects had to push the ball to half-maximal contraction as previously shown.

Safety

Neuron-specific enolase determination

To assess the safety of tRNS, we measured serum neuron-specific enolase (NSE), a sensitive marker of neuronal damage, evident in many neurological disorders, e.g., in epilepsy (Steinhoff et al., 1999). Elevated NSE concentration is a specific marker in intractable temporal lobe epilepsy. A blood sample for NSE-measurement was taken in six healthy subjects before and 10 min after stimulation. Furthermore, in one subject, who was stimulated for 8 consecutive days, this measurement was done on every day.

EEG recording

The EEG was recorded using a three-channel montage. One electrode was placed over Oz and two laterally above the motor region (C3 and C4) in accordance with the international 10/20 system. The impedance was kept at <5 kΩ. Linked mastoids (RLm) were used as a reference. The ground electrode was positioned on the forehead. Data were collected with a sampling rate of 1000 Hz using BrainAmp system (Brain Products) and were analyzed off-line (Brain Vision Analyzer, Brain Products).

The EEG experiments were conducted in a repeated-measurement design (tRNS and sham) using a randomized order, with a minimum break of 1 d between each stimulation session. Two minutes EEG was recorded at rest before and three times after stimulation (immediately and 7 and 14 min after the end of the stimulation). EEG epochs (2 min) were segmented for 30 s and filtered by using 0.1 Hz (24 dB/octave) low cutoff and a 70 Hz (24 dB/octave) high cutoff and 50 Hz notch filters. In addition to semiautomatic artifact detection (200 μV amplitude criterion), all epochs were visually inspected, and those containing eye blinks or muscle movement artifacts were excluded. After artifact rejection, all of the epochs were segmented into 2 s, and fast Fourier transformation (FFT) was calculated for all electrodes (0.5 Hz resolution, and 10% Hamming window). The FFT segments were averaged for each 30 s. The mean activity in voltage was calculated and exported from each frequency bands (theta band 4.5–7 Hz, alpha band 8–12 Hz, beta band 12.5–30 Hz, and gamma band 31–49 Hz) for statistical analysis.

For sham stimulation, the current was turned on for 30 s at the beginning of the stimulation. Subjects were blinded for stimulation conditions in all of the studies.

Data analyses

Electrophysiological studies

Single-pulse TMS. Repeated measurements of ANOVAs [condition (tRNS vs sham) $\times$ time (before; 0, 5, 10, 20, 30, 40, 50, 60 min after stimulation; ($n = 8$: before; 0, 5, 10, 20, 30, 40, 50, 60, 90 min and 2, 4, 6, 24 h after stimulation)] were used to compare the different conditions. Effects were considered significant if $p < 0.05$. In the case of a significant interaction of time and stimulation condition, a Tukey's *post hoc* test was performed. Student's *t* test was used to compare the motor thresholds (RMT, AMT, and SI1mV) between experimental sessions. All data are given as means $\pm$ SEM.

Paired-pulse TMS. For each measurement (SI1mV, RMT, AMT, SICI, ICF, LICI, and CSP), we performed separate ANOVAs for repeated measurements by using the mean values from each subject as the dependent variable. In addition to the factor "stimulation type" (tRNS vs sham), the ANOVA model included the factor "ISI" (2, 4, 7, 9, 12, 15, and 25 ms) when SICI and ICF was analyzed, the factor "intensity" (100%, 130%, and 150% of RMT) for recruitment curves, or the factor "intensity" (120% RMT and SI1mV) for CSP. A *p* value of <0.05 was considered significant for all statistical analyses. In the case of a significant interaction between ISI/intensity and stimulation condition, a Tukey *post hoc* test was performed. Student's *t* test was used to compare the motor thresholds (RMT, AMT, and SI1mV) between experimental sessions. Data are expressed as mean $\pm$ SEM.

Behavioral studies

SRTT analysis. Concerning the implicit learning paradigm, statistical analysis was performed with repetitive-measures ANOVA (independent variables current condition and block) for reaction time (RT), error rate (ER), and variability. As the RT and ER differences between blocks 5 and 6 are thought to represent an exclusive measure of implicit learning, interactive Student's *t* tests were performed to compare the respective differences between tRNS and sham conditions. In each trial, RT was measured from the appearance of the "go" signal until the first button was pushed by the subject. For each block of trials of a given experimental condition, mean RT was calculated for each subject separately. Furthermore, as a measure of the variability of the RTs, we have calculated the coefficient of variation (the ratio of the SD to the mean $\times$ 100). An ER was calculated to assess the number of incorrect responses for each block and each subject in each stimulation condition.

Task-related modulation of tRNS. Repeated-measures ANOVA [experiment (passive vs cognitive/motor) $\times$ time (before and 5, 10, 15, 20, 25, and 30 min after stimulation, then every 15 min up to 2 h)] was used to compare different task conditions during tRNS. Effects were considered significant if $p < 0.05$. In case of the significant interaction of time and stimulation condition, a Tukey *post hoc* test was performed. Student's *t* test was used to compare the motor thresholds (RMT, AMT, and SI1mV) between experimental sessions. All data are given as means + SEM.

Safety

NSE determination. Two-tailed *t* tests (paired samples, critical *p* value 0.05) were performed to compare NSE values before and after tRNS.

EEG recording. To compare the effect of stimulation on the EEG spectrum, a repeated-measures ANOVA (independent variable: tRNS vs sham $\times$ time points after stimulation; dependent variable: FFT power in a given frequency band) was calculated.

Results

All of the subjects tolerated the stimulation; none of the experimental sessions were interrupted due to side effects of the stimulation. Only two of 80 subjects reported a slight burning sensation under the electrodes during the stimulation.

Table 1. Results of the statistical analyses in the case of the single- and paired-pulse TMS studies over the primary motor cortex

	Measurement	Factor	df	F/t^a	p
Single-pulse TMS					
Student's *t* test	RMT		10	0.90	0.39
	AMT		10	1.68	0.12
	SI1mV		10	0.42	0.69
ANOVA		Condition	1	7.24	**0.01**
		Time	28	4.01	**<0.01**
		Condition × time	28	3.53	**<0.01**
Paired-pulse TMS					
Student's *t* test	RMT		9	0.42	0.68
	AMT		9	0.90	0.39
	SI1mV		9	0.01	1.00
ANOVA		Condition	1	0.80	0.39
		Intensity	2	19.03	**<0.01**
	RECR	Condition × intensity	2	0.38	0.69
		Condition	1	0.38	0.54
		ISI	1	47.94	**<0.01**
	SICI	Condition × ISI	1	0.13	0.73
		Condition	1	0.58	0.46
		ISI	3	0.88	0.46
	ICF	Condition × ISI	3	5.56	**<0.01**
		Condition	1	0.23	0.64
		ISI	4	4.04	0.01
	LICI	Condition × ISI	4	0.37	0.83
		Condition	1	0.63	0.44
		Intensity	1	1.05	0.33
	CSP	Condition × intensity	1	0.81	0.38

RECR, Recruitment curves. Bold indicates significant values. [a]*F* for ANOVA and *t* for Student's *t* test.

Electrophysiological studies: MEPs

Single-pulse TMS

When 10 min tRNS was applied over the primary motor cortex, the induced excitability increases rose up to 20–50%, as revealed by TMS. They last for 60 min after stimulation. Repeated measurements of ANOVA revealed a significant main effect of condition ($F_{(1,28)} = 7.24$, $p = 0.01$) and time ($F_{(8,224)} = 4.01$, $p < 0.001$) in the case of motor cortex stimulation. The interaction between condition and time was also significant ($F_{(8,224)} = 3.53$, $p < 0.001$) (Table 1). According to the *post hoc* analysis, significantly increased MEPs were observed at the 5 and 10–60 min time points compared with the time point before ($p < 0.05$) tRNS (Fig. 2).

RMT, AMT, and SI1mV baseline values were compared between RN and sham stimulation conditions using Student's *t* test. There was no significant difference between tRNS and sham stimulation in any of the measurements (Table 1).

Furthermore, we have separated the stimulation spectrum into low (0.1–100 Hz)- and high (101–640 Hz)-frequency ranges. High-frequency stimulation was more effective with regard to changing the level of cortical excitability. Repeated measurements of ANOVA revealed a significant effect of condition ($F_{(1,21)} = 4.2$, $p = 0.05$) when the high-frequency spectrum stimulation was used, compared with the sham condition. However, there was no significant effect of condition, when the low-frequency spectrum was applied ($F_{(1,20)} = 2.22$, $p = 0.15$). There was no significant condition × time interaction ($F_{(7,147)} = 1.62$, $p = 0.13$ and $F_{(7,140)} = 0.78$, $p = 0.61$, respectively) (Fig. 3).

We did not observe any changes in corticospinal excitability when the premotor cortex was stimulated, implying that the effect of tRNS over the M1 is indeed focal. Repeated measurements of ANOVA revealed no significant effect on condition ($F_{(1,18)} = 0.01$, $p = 0.99$) nor time ($F_{(8,14)} = 0.78$, $p = 0.61$). There was no significant condition × time interaction ($F_{(8,14)} = 0.69$, $p = 0.70$).

The possibility of a hidden DC shift in the stimulation spectrum as a cause of the excitability increase was excluded by the results of a control experiment conducted by reversing the connection of the electrodes to the stimulator. In the case of measuring DC-shift-induced excitability changes, repeated measurements of ANOVA revealed no significant effect of condition ($F_{(1,14)} = 0.29$, $p = 0.60$). The effect of time was significant ($F_{(8,112)} = 2.13$, $p = 0.04$). There was no significant condition × time interaction ($F_{(8,112)} = 0.24$, $p = 0.98$).

Paired-pulse TMS

In our paired-pulse TMS study, we have observed an increase in ICF after tRNS over M1. Repeated measurements of ANOVA revealed no significant effect of condition ($F_{(1,9)} = 0.58$, $p = 0.46$) or ISI ($F_{(3,27)} = 0.88$, $p = 0.46$). However, the interaction between condition and ISI was significant ($F_{(3,27)} = 5.56$, $p = 0.004$). According to the *post hoc* analysis, significantly increased MEPs were observed at ICF of 12 and 15 ms after tRNS compared with the sham condition ($p < 0.05$).

tRNS administration had no effect on SICI, LICI, CSP, or motor-evoked recruitment curves as revealed by repeated measurements of ANOVA (Table 1).

Behavioral studies

SRTT

With regard to the functional effect of tRNS, it significantly improved performance in the acquisition and early consolidation phase of motor learning. This was primarily represented by the differences between blocks 5 and 6 between tRNS and sham conditions, which are exclusive measurements of implicit learning. Compared with the sham stimulation condition, RTs in the SRTT shortened during tRNS of the primary motor cortex; and subjects became faster during the course of the experiment.

Repeated-measures ANOVA revealed a significant effect on blocks ($F_{(7,112)} = 37.59$, $p < 0.001$). This was caused by an interaction of tRNS versus sham stimulation for block 5 and block 6, due to a greater difference in the case of tRNS ($t = -2.87$, df = 16, $p = 0.01$) as revealed by Student's *t* tests. There was no significant effect on stimulation. However, the stimulation × blocks interaction was marginally significant ($F_{(7,112)} = 1.95$, $p = 0.06$). Figure 4 shows the differences between RN and sham stimulation. The paradigm was repeated in six subjects after 1 and 2 h after stimulation. At these time points the RTs were not significantly different between the tRNS and sham stimulation conditions (see Fig. 4). However, the RTs of the sham and tRNS trials were not the same as those observed after the familiar blocks immediately after stimulation, but are the same after 1 h; the control RTs decreased substantially in the 1 h period for the familiar block, which may represent consolidation of learning, whereas this was not the case for the tRNS group. Nevertheless, only six subjects were analyzed after 1 h.

For the ER, the ANOVAs showed a significant main effect on

blocks ($F_{(7,112)} = 2.54$, $p = 0.02$). Despite this, the results of all other tests remained insignificant. Student's t tests revealed no significant difference between blocks 5 and 6. For RT variability, the ANOVAs showed a significant main effect on blocks ($F_{(7,112)} = 29{,}12$, $p < 0.0001$) without significant interaction between condition and blocks.

Task-related modulation of tRNS

Excitability increase induced by tRNS was modified by paying attention to a task involving mental activity and by contraction of the target muscle during the stimulation. Following tRNS, the amplitude of the MEPs was increased in the passive condition, slightly decreased in the cognitive condition and markedly reduced in the motor condition. When the amplitude of the MEPs was compared with regard to the passive condition and cognitive task before and after stimulation, repeated-measures ANOVA revealed a main effect of experiment ($F_{(1,11)} = 5.45$, $p = 0.04$), but time ($F_{(12,132)} = 0.50$, $p = 0.91$) was not significant. The interaction between the experiment and time was significant ($F_{(12,132)} = 2.36$, $p = 0.009$). The *post hoc* test revealed that, after tRNS in the passive condition, significantly increased MEP amplitudes were observed up to 20 min, and at the 1 and 2 h time points when compared with the cognitive task condition ($p < 0.01$). When the amplitude of the MEPs was compared with the passive condition and motor task, repeated measures of ANOVA revealed a main effect of experiment ($F_{(1,11)} = 10.05$, $p = 0.009$), but time ($F_{(12,132)} = 0.74$, $p = 0.71$) was not significant. The interaction between the experiment and time was significant ($F_{(12,132)} = 3.96$, $p < 0.001$). The *post hoc* test revealed that, after tRNS in the passive condition, significantly increased MEP amplitudes were observed up to 25 min ($p < 0.01$) compared with the motor condition.

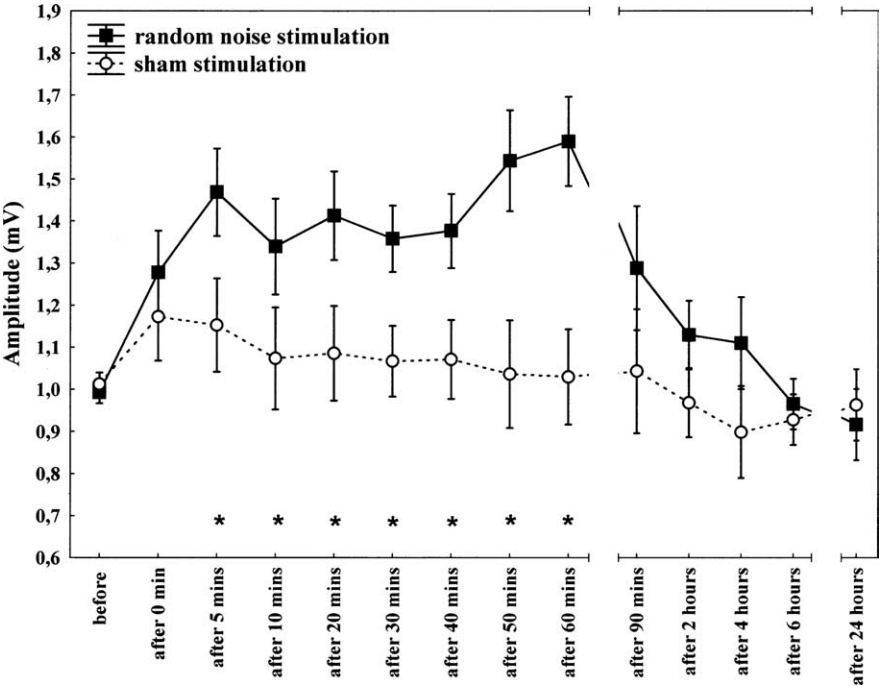

Figure 2. Effect of 10 min RN stimulation on motor-evoked potentials. Time course of motor cortex excitability changes lasting for 60 min after stimulation, shown after 10 min RN stimulation over M1 at 1 mA compared with sham stimulation. The figure shows mean amplitudes and their SEMs up to 60 min (including all subjects, $n = 17$) and between 90 min and 24 h (including 8 subjects). Asterisks indicate significant differences between MEP amplitudes after 5 and 10–60 min after stimulation and those at baseline.

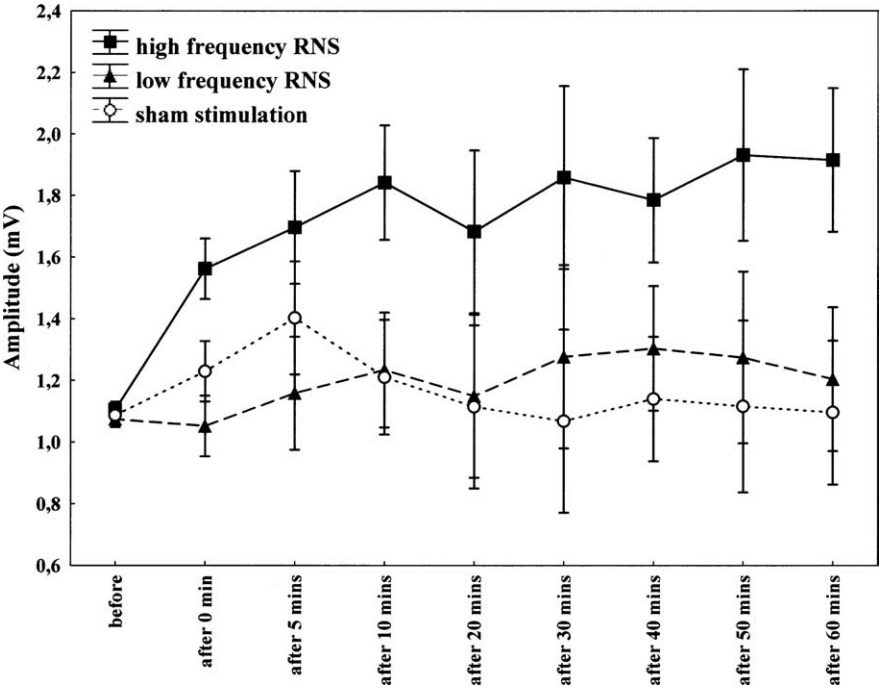

Figure 3. Effect of 10 min of low (0.1–100 Hz)- and high (101–640 Hz)-frequency RN stimulation on motor-evoked potentials. Time course of motor cortex excitability changes lasting for 60 min after stimulation, shown after 10 min of high-frequency RN stimulation over M1 at 1 mA compared with low-frequency and sham stimulation. The figure shows mean amplitudes and their SEMs up to 60 min (including all subjects, $n = 12$).

Safety

The concentration of serum NSE was unchanged after tRNS. Student's t test showed no significant difference between the before and after stimulation NSE concentrations of six healthy subjects ($t = 0.09$, $p = 0.93$, mean value before stimulation: 6.96 ± 1.84 μg/L, after stimulation: 6.91 ± 1.7 μg/L). One subject was stimulated for 10 min every day for 8 consecutive days. The NSE values did not change significantly over the period from the first to last day of stimulation ($t = -0.2$, $p = 0.87$, mean value before stimulation: 9.57 ± 2.2 μg/L, after stimulation: 9.53 ± 3.0 μg/L).

Furthermore, we recorded EEGs before and after tRNS and did not find any significant difference regarding any of the frequency bands. Repeated-measures ANOVA revealed no significant interactions between current conditions, time, or channels for any of the different frequencies applied (see supplemental

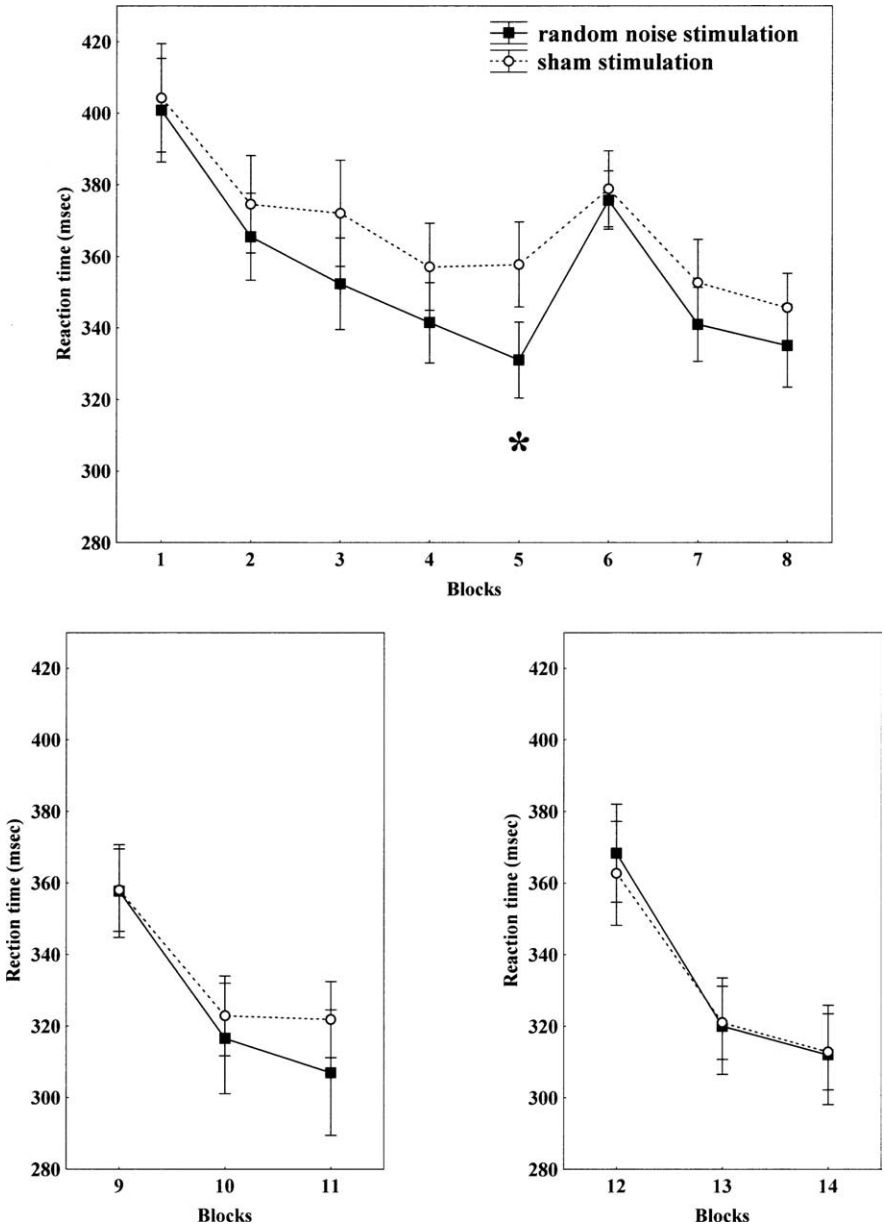

Figure 4. tRNS of the primary motor cortex improves implicit motor learning in its early phase. Reaction times decrease faster in the tRNS condition when compared with the sham stimulation condition (top). Moreover, the RT difference comparing blocks 5 and 6, which indicates implicit sequence learning, is bigger for the tRNS condition, when compared with sham condition. The asterisk indicates a significant difference regarding reaction time differences between blocks 5 and 6 between RN and sham stimulation. In 1 and 2 h after stimulation, this significant difference was no longer detectable (bottom panels).

Table 2, available at www.jneurosci.org as supplemental material). Additionally, we did not see any abnormal EEG activity after tRNS. Therefore, we can conclude that limited exposure to tRNS of the cortex using the parameters we applied here is safe.

Discussion

In this study, we demonstrate that weak tRNS over M1 enhances corticospinal excitability both during and after stimulation in the healthy human brain. Furthermore, our results suggest that the high-frequency subdivision of the whole tRNS spectrum between 100 and 640 Hz is functionally responsible for inducing excitability in the M1. In terms of commonly used noninvasive excitability parameters, we found an increased ICF after tRNS over M1 using the paired-pulse paradigm. tRNS application had no effect on SICI, LICI, CSP, or motor-evoked recruitment curves [for an overview of methods used to study the modulation of human motor cortex excitability in local circuits, see Paulus et al. (2008) and Ziemann et al. (2008)]. Pharmacological studies show that among others, ICF is most likely to be mediated by the glutamatergic system (Ziemann et al., 1998), possibly by the activation of glutamatergic synapses by tRNS. However, no clear evidence was found concerning the cortical origin of ICF, in a recent study in which epidural recording was applied in a conscious subject (Di Lazzaro et al., 2006). The results of this study showed that, despite a significant increase in MEP at ISIs of 10 and 15 ms, there is no evident change in the descending volley. Thus at ISIs of 10 and 15 ms, a small conditioning stimulus can produce clear facilitation of MEPs even though it leads to no detectable change in descending corticospinal activity.

The average MEP decrease observed after mental effort and motor activation are in agreement with previous studies using tDCS (Antal et al., 2007) and paired associative stimulation (PAS) (Stefan et al., 2004). Similarly, a recent study observed that contraction of the FDI muscle during TBS abolished the effects of stimulation on the MEPs (Huang et al., 2008). These results suggest that externally induced neuronal plasticity is highly dependent on the state of the subject during stimulation.

It appears that the tRNS-driven cortical excitability change facilitates the learning process. Previous studies suggest that an excitability enhancement coincides with facilitating the learning process by inducing the strengthening of synapses and inducing long-term potentiation via modifying NMDA-receptor efficacy (Rioult-Pedotti et al., 2000). Regarding studies in the human, this is in line with previous observations of increased activation of the M1 during motor learning tasks (Grafton et al., 1992; Honda et al., 1998), showing that effects of motor training can be improved by cortical excitability enhancements. Additionally, our results describing an increase in corticospinal excitability and facilitating learning with regard to the SRTT more closely resemble those reported by previous studies after anodal tDCS (Nitsche and Paulus, 2000, 2001); even more so, since we applied well proven tDCS parameters such as electrode position, intensity, and stimulation duration.

There is, however, a key difference between tDCS and tRNS. tDCS modifies the transmembrane neuronal potential directly and thus modulates the firing rate of individual neurons (Bindman et al., 1964). In contrast, the stimulation spectrum of tRNS does not possess a DC component. In addition, the physiological control experiment conducted by reversing the electrode position did not influence the characteristic excitability-enhancing

aftereffect, in contrast to the inhibition that we see with cathodal tDCS (Nitsche and Paulus, 2000). Several physiological mechanisms may underlie the tRNS effects. tRNS, like alternating current stimulation (tACS) (Antal et al., 2008), can possibly interfere with ongoing oscillations and neuronal activity in the brain and thus result in a cortical excitability increase. However, tACS with intensities of >400 μA (Antal et al., 2008) induced a flickering sensation via retinal stimulation, and as a result [at least in the frequency range that we applied (1–45 Hz)], we were reluctant to increase the intensity further, at least with the standard reference montage at the forehead close to the retina. Also, the tACS type of monophasic sinusoidal stimulation is more likely to be epileptogenic than that of a random noise waveform. For this reason, we started by using a random noise frequency spectrum with a range of 0.1–640 Hz; the latter frequency is known to represent the high end of physiologically measured human electric brain oscillations (Gobbelé et al., 2000).

We did not make current density calculations of how effectively the high-frequency component of the stimulus is transmitted to the brain. There is, however, sufficient evidence to suggest that the current used here can reach the brain. The bone is the structure with highest resistance, and has to be considered primarily when stimulating the head electrically (Wagner et al., 2008). In fact, high bone resistance was the reason why TMS replaced pulsed electrical stimulation in 1985 (Barker et al., 1985) and thereby could avert painful stimulation. It was found that the bone conductivity on the three orthogonal directions was constant up to 10 kHz (Reddy and Saha, 1984) even above the range of the frequencies used in our study. Distinctly higher frequencies of 50 kHz could still pass through the skull as measured by electrical impedance tomography (Abascal et al., 2008). The dielectric properties of bone was shown to be constant between frequencies of 10 and 100,000 Hz (Gabriel et al., 1996).

A previous study by Yamamoto et al. (2005) used a distinctly lower frequency range (<2 Hz) in patients with Parkinson's disease (PD). Their method, however, differed from ours in electrode position, stimulation amplitude, duration, and techniques of evaluation. Improved autonomic and motor functions were detected after 24 h of continuous noisy vestibular electrical stimulation over the bilateral mastoids. The authors hypothesized that in PD patients the input noise ameliorated the impaired neuronal transmission, and the noise itself enhanced weak neuronal signal detection in the sensory system; the phenomenon of stochastic resonance, as shown in several experimental studies (e.g., Moss et al., 2004). Indeed, it has been suggested that noisy electrical fluctuations can boost synaptic signals.

Stochastic resonance may play a role in tRNS, however at much higher frequency ranges. For some years now, oscillations within a frequency range of 80–200 Hz (ripples) have been associated with plasticity processes (Grenier et al., 2001) and learning (Ponomarenko et al., 2008). Another putative mechanism of tRNS may be activation of sodium channels via rectification by high-frequency stimulation (Bromm, 1968). The postulated tRNS effect begins with the depolarization of a neuronal membrane which causes Na^+ channels to open. This allows an influx of Na^+ ions to flow down the concentration gradient and increases membrane depolarization. If the Na^+ entry is insufficient, there is no regenerative depolarization and thus no action potential, just the "local response." The repolarization occurs passively over a longer period of time compared with the duration of Na^+ ion entry. If stimulation is repeated, the Na^+ channels can reopen and induce a second Na^+ ion influx, which depolarizes the membrane further, heightening the effect of the preceding depolarization. The Na^+ channels then close, and after repolarization can be reopened by succeeding depolarizations. Indeed, recently it was shown that repetitive extracellular high-frequency stimulation in cultured rat neurons activated an inward sodium current, which gave rise to a weak depolarization of the cell membrane (Schoen and Fromherz, 2008). Although the time integral of the stimulating current used in a voltage clamp study was zero, the average membrane potential was shifted in the direction of depolarization. The resulting depolarization was understood to be the result of the nonlinearity of the sodium current–voltage input during subthreshold excitation. Since we used a symmetric high-frequency stimulation, this nonlinearity could be the reason for the excitatory effects we have seen with tRNS. Interestingly, the effect of tRNS increased with time after stimulation. Effects induced by "repetitive activation of Na^+ channels by weak capacitive currents" studied by Schoen and Fromherz (2008) also increase with stimulation time, however within a much shorter time range (<1 s). On the other hand, continuous opening of Na^+ channels would lead to membrane depolarization, from which we can assume from previous tDCS studies that a time range of >3 min may lead to LTP-like mechanisms. However, the neuronal membrane is a more intricate structure and possesses numerous voltage-gated ion channels and is subject to simultaneous influxes of ionic currents (Ca^{2+}, K^+, etc). Indeed, because the membrane is encumbered with multiple voltage-gated channels, that are "nonlinear," the aforementioned induced changes in membrane fluctuation can be amplified. In summary, a pure DC stimulus can open Na^+ channels just once, whereas repeated pulses (tRNS) can induce multiple ionic influxes, and achieve substantially heightened effects. The interval at which the pulses are repeated must be short and relates to the time constant of the nerve membrane.

Thus, finally, the neuroplastic effects of tRNS could be analogous to anodal tDCS aftereffects, but with clear advantages. tRNS can circumvent problems that can arise by stimulating a folded cortex with anodal stimulation, since on one side of the gyrus wall current orientation induces excitation, while on the opposite side of the gyrus, it will inevitably induce inhibition. When using tRNS only excitatory aftereffects are observable. Also "tangential" stimulation of nerve cells now appears to be possible with tRNS. Within a "tangential" DC electric field applied to a symmetrical dendritic arbor, currents on both sides would cancel each other at the axon hill. In the case of a rectifying depolarization using a fast oscillating field, the cell would be depolarized regardless of current flow orientation. Safety concerns are probably lessened than in the case of tDCS. Several anecdotal, but so-far-unpublished, reports have described small skin burns after tDCS. In general, nonpolarizing currents seem to be safer than polarizing currents as seen in deep brain stimulation. Here we have not observed any tRNS-induced changes with EEG recordings (see supplemental Table 2, available at www.jneurosci.org as supplemental material). tRNS using 1 mA was unnoticed in 78 of 80 subjects, compared with a slight skin tingling sensation with tDCS. Thus it appears to have the best blinding potential for controlled studies of currently available methods.

In summary, tRNS allows an unnoticeable and thus painless, selective, focal, noninvasive, and reversible excitability increase of the cortex. Apart from being more economically viable than rTMS its main advantage seems to be the direction insensitivity of the stimulation. It seems to provide a qualitatively new way of producing and interfering with brain plasticity.

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Towards unravelling task-related modulations of neuroplastic changes induced in the human motor cortex

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Keywords: motor contraction, motor evoked potential, transcranial direct current stimulation, transcranial magnetic stimulation

Abstract

Stimulation with weak electrical direct currents has been shown to be capable of inducing stimulation-polarity-dependent prolonged diminutions or elevations of cortical excitability, most probably elicited by a hyper- or depolarization of resting membrane potentials. The aim of the present study was to test if cognitive task and motor exercise practiced during the stimulation are able to modify transcranial direct current stimulation-induced plasticity in the left primary motor cortex in 12 healthy subjects. Motor evoked potentials were recorded before and after 10 min of anodal and cathodal transcranial direct current stimulation. In Experiment 1, subjects were required to sit passively during the stimulation, in Experiment 2 the subject's attention was directed towards a cognitive test and in Experiment 3 subjects were instructed to push a ball in their right hand. Both the cognitive task and motor exercise modified transcranial direct current stimulation-induced plasticity; when performing the cognitive task during stimulation the motor cortex excitability was lower after anodal stimulation and higher after cathodal stimulation, compared with the passive condition. When performing the motor exercise, the motor cortex excitability was lower after both anodal and cathodal stimulation, compared with the passive condition. Our results show that transcranial direct current stimulation-induced plasticity is highly dependent on the state of the subject during stimulation.

Introduction

Transcranial direct current stimulation (tDCS) appears to be a promising tool in neuroplasticity research with perspectives in clinical neurophysiology (Fregni & Pascual-Leone, 2007). Its effect is closely related to modulation of cortical excitability and activity, which are key mechanisms for learning and memory processing (Paulus, 2004). The primary effect of tDCS is a neuronal de- or hyperpolarization of membrane potentials (Creutzfeldt *et al.*, 1962; Bindman *et al.*, 1964), whereby the induced after-effects depend on *N*-methyl-D-aspartate (NMDA) receptor efficacy changes (Liebetanz *et al.*, 2002). There is also evidence for both GABAergic (Nitsche *et al.*, 2004a) and dopaminergic modulation of tDCS-induced effects (Nitsche *et al.*, 2006). The most common way to evaluate cortical excitability changes is by applying transcranial magnetic stimulation (TMS) to the motor cortex, as it allows reproducible and quantifiable effects through the analysis of motor evoked potentials (MEPs). Anodal stimulation increases the amplitude of MEPs and cathodal stimulation decreases them (Nitsche & Paulus, 2000). The relevant stimulation parameters encompass the polarity, the combination of current strength, size of the stimulated area and duration of the stimulation (Agnew & McCreery, 1987) and are considered to be safe by several studies (Nitsche *et al.*, 2003; Iyer *et al.*, 2005; Poreisz *et al.*, 2007).

The aim of this study was to investigate whether a mental or motor activity performed during stimulation can modify the efficacy of tDCS. Therefore, the subjects were required to pay attention and fill out a cognitive task or push a ball for the duration of the anodal or cathodal tDCS.

A recent study applied a paired associative stimulation (PAS) protocol (Stefan *et al.*, 2004). PAS-induced changes of cortical excitability, similarly to tDCS-induced plasticity, share a number of physiological properties with LTP (for a review see Classen *et al.*, 2004). PAS-induced plasticity was completely blocked when the subject's attention was directed toward a cognitive test.

With regard to motor task, several previous studies have examined the effect of motor exercise and related muscle fatigue on corticospinal activity. Motor fatigue is defined as a reduction in the force generated by a muscle or a group of muscles after sustained or repeated contraction (Merton, 1954). In recent years the central component of fatigue was extensively investigated using TMS (Gandevia, 1996; Samii *et al.*, 1996; Sacco *et al.*, 1997, 2000; Zijdewind *et al.*, 2000) and it was shown that, immediately after a non-exhaustive exercise, the amplitude of TMS-induced MEPs increases (Balbi *et al.*, 2002). If the exercise is repeated until muscle fatigue, a MEP amplitude decrease can be observed (Brasil-Neto *et al.*, 1994).

Furthermore, attentional and cognitive deficits and involuntary motor contractions are frequent symptoms of many neurological and psychiatric disorders such as Alzheimer's disease (Claus & Mohr, 1996), Huntington's disease (Sprengelmeyer *et al.*, 1995; Finke *et al.*, 2006) and Parkinson's disease (Claus & Mohr, 1996; Braak *et al.*, 2005). If the efficacy of tDCS in inducing motor cortical excitability is a task-related parameter, the magnitude of plasticity might be variable or even completely blocked in patients compared with healthy subjects.

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Materials and methods

Subjects

Twelve healthy volunteers (six males; aged between 21 and 26 years, mean age 22.75 ± 1.36 years) were informed about all aspects of the experiments and all signed an informed consent form. All were consistent right-handers according to the 10-item version of the Edinburgh Handedness Inventory (Oldfield, 1971). We conformed to the Declaration of Helsinki and the experimental protocol was approved by the Ethics Committee of the University of Göttingen. None of the subjects suffered from any neurological or psychological disorders and none had metallic implants/implanted electric devices or took any medication regularly.

Transcranial direct current stimulation

Direct currents were transferred via a pair of saline-soaked surface sponge electrodes (5 × 7 cm) fixed to the scalp and delivered by a specially developed battery-driven current stimulator (NeuroConn GmbH, Ilmenau, Germany). The motor-cortical electrode was placed over the representational field of the right first dorsal interosseus muscle (FDI) as identified by TMS, whereas the other electrode was located contralaterally above the right eyebrow. The electrodes were orientated approximately parallel to the central sulcus and the eyebrow. This montage has been proven to be the most effective for modulating motor cortex excitability (Nitsche & Paulus, 2000; Nitsche *et al.*, 2003). The type of stimulation (anodal or cathodal) refers to the polarity of the electrode above the motor cortex. Subjects were blinded as to the polarity of tDCS. The current was applied for 10 min with an intensity of 1.0 mA. The fade-in/fade-out time was 8 s.

Transcranial magnetic stimulation

To detect current-driven changes of excitability, MEPs of the right FDI were recorded following stimulation of its motor-cortical representational field by single-pulse TMS. These were induced using a Magstim 200 magnetic stimulator (Magstim Company, Whiteland, Wales, UK) and a figure-of-eight standard double magnetic coil (diameter of one winding, 70 mm; peak magnetic field, 2.2 T; average inductance, 16.35 μH). The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline, resulting in a posterior–anterior direction of current flow in the brain. The optimal position was defined as the site where stimulation resulted consistently in the largest MEP. The site was marked with a skin marker to ensure that the coil was held in the correct position throughout the experimental sessions. Surface EMG was recorded from the right FDI by use of an Ag/AgCl electrode in a belly tendon montage. The signals were amplified and filtered (2 Hz–3 kHz; maximal signal frequency, 1 kHz; sampling rate, 5 kHz), digitized with a micro 1401 AD converter (Cambridge Electronic Design, Cambridge, UK) and recorded by a computer using Signal software (Cambridge Electronic Design, version 2.13). Data were analysed offline on a personal computer. Complete muscle relaxation was controlled though auditory and visual feedback of EMG activity. The intensity of the stimulator output was adjusted for baseline recording so that the average stimulus led to an MEP of ~1 mV (SI_{1mV}).

Experimental procedures

The six experimental sessions were conducted in a repeated measurement design using a randomized order, with a break of at least 4 days between each session. The subjects were seated in a reclining chair. First, the left motor-cortical representational field of the right FDI was identified using TMS (coil position that leads to the largest MEPs of FDI). After determining the resting and active motor thresholds, the subjects were asked to relax for at least 5 min. A baseline of TMS-evoked MEPs (50 stimuli) was then recorded at a time constant of 4 ± 0.04 s. Afterwards, one direct current stimulation electrode, in an anodal or cathodal orientation, was fixed at the representational field of the right FDI and the other was fixed at the contralateral forehead above the orbita.

During anodal and cathodal stimulation, subjects were passively sitting during the stimulation (Experiment 1), had their attention directed towards a cognitive test (Experiment 2) or were instructed to push a ball in their right hand (Experiment 3). After termination of tDCS, a 5 min break was inserted into the protocol as the pilot experiments indicated that many of the subjects were not able to relax after the termination of the motor exercise. After this break 25 MEPs were recorded at a time constant of 4 ± 0.04 s every fifth minute up to 30 min (in the case of 12 subjects) and then every 15 min up to 90 min (in the case of six subjects).

During the stimulation in Experiment 2 the subjects were required to fill out a cognitive test that was presented on a computer monitor. The subjects had to push a suitable button with their right index finger in order to give the correct answer. The test was presented in German and downloaded from a commercial intelligence test homepage. To avoid any training effect we used a cognitive task with a different series of questions during the anodal and cathodal tDCS. The questions were on a variety of subjects, i.e. mathematics, literature, geography and history, and were all of different lengths; therefore, a direct comparison of the results was not possible (the number of responses was measured instead of reaction time). However, the accuracies and the subjective reports of the subject were documented after stimulation.

In Experiment 3, the subjects were instructed to push a ball (8 cm diameter) in their right hand. The ball was connected to a display where the actual values related to pressure were quantified. Before the stimulation session the subjects were asked to push the ball as hard as possible. During the tDCS session subjects had to push the ball to half-maximal contraction as previously shown.

Statistical analysis

Repeated measures ANOVA [EXPERIMENT (passive vs. cognitive/motor) × TIME (before, 5, 10, 15, 20, 25 and 30 min after stimulation] was used to compare different task conditions during anodal or cathodal stimulation. Effects were considered significant if $P < 0.05$. Bonferroni corrected *t*-test was used for post-hoc comparison. Student's *t*-test was used to compare the motor thresholds (resting motor threshold, active motor threshold and SI_{1mV}) between experimental sessions. All data are given as means + SEM.

Results

All of the subjects tolerated tDCS and had no side-effects during or after the stimulation.

Active motor threshold, resting motor threshold and SI_{1mV} baseline values were compared between anodal and cathodal conditions within the passive condition, and concerning the cognitive and motor tasks using Student's *t*-test. There was no significant difference between anodal and cathodal stimulation in any of the measurements at baseline.

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A subjective decline in performance was reported by two of the subjects in the case of anodal tDCS in Experiment 2.

Anodal stimulation

Following anodal stimulation the amplitude of the MEPs was increased in the passive condition, slightly decreased in the cognitive condition and markedly reduced in the motor condition. When the amplitude of the MEPs was compared with regard to the passive condition and cognitive task before and after anodal stimulation, repeated measures ANOVA revealed a main effect of EXPERIMENT ($F_{1,11} = 9.25$, $P = 0.01$) but TIME ($F_{6,66} = 1.89$, $P = 0.09$) and the interaction between EXPERIMENT and TIME were not significant ($F_{6,66} = 1.55$, $P = 0.17$). When the amplitude of the MEPs was compared with regard to the passive condition and motor task, repeated measures ANOVA revealed a main effect of EXPERIMENT ($F_{1,11} = 39.46$, $P < 0.0000$) but TIME was not significant ($F_{6,66} = 0.9$, $P = 0.49$). The interaction between EXPERIMENT and TIME was significant ($F_{6,66} = 6.77$, $P < 0.0001$). The post-hoc test revealed that, after anodal stimulation in the passive condition, significantly increased MEP amplitudes were observed up to 25 min ($P < 0.0001$) (Fig. 1).

Cathodal stimulation

Following cathodal stimulation the amplitude of the MEPs was decreased in the passive condition, slightly increased in the cognitive condition and markedly diminished in the motor condition. When the amplitude of the MEPs was compared with regard to the passive condition and cognitive task before and after cathodal stimulation, repeated measures ANOVA revealed a main effect of EXPERIMENT ($F_{1,11} = 52.44$, $P < 0.0000$) and TIME ($F_{6,66} = 3.57$, $P = 0.004$). The interaction between EXPERIMENT and TIME was also significant ($F_{6,66} = 4.23$, $P = 0.001$). The post-hoc test revealed that, after cathodal stimulation in the passive condition, significantly increased MEP amplitudes were observed up to 30 min ($P < 0.03$) (Fig. 1). When the amplitude of the MEPs was compared with regard to the passive condition and motor task, repeated measures ANOVA revealed a main effect of EXPERIMENT ($F_{1,11} = 12.59$, $P < 0.04$) and TIME ($F_{6,66} = 20.09$, $P < 0.0000$). The interaction between EXPERIMENT and TIME was not significant ($F_{6,66} = 1.69$, $P = 0.13$) (Fig. 1).

Discussion

To our knowledge, the present study is the first to show that neuronal plasticity induced in the human primary motor cortex by tDCS is modified by paying attention to mental activity and by repeated contractions of the target muscle during the stimulation. In the passive condition, anodal stimulation increased whereas cathodal stimulation decreased the amplitude of MEPs, as described in many previous studies (Nitsche & Paulus, 2000, 2001; Lang *et al.*, 2004). However, when the subjects were required to perform a cognitive test during stimulation, the MEP amplitudes were slightly increased after cathodal stimulation or were non-significantly decreased after anodal stimulation. Voluntary motor contraction during anodal and cathodal stimulation resulted in a decrease in MEP amplitudes, probably due to muscle fatigue as described by many previous studies (Samii *et al.*, 1996; Sacco *et al.*, 1997, 2000; Zijdewind *et al.*, 2000), independent of the polarity of the stimulation.

A recent study by Stefan *et al.* (2004) applied a similar paradigm, but using a different method, in order to describe attentional

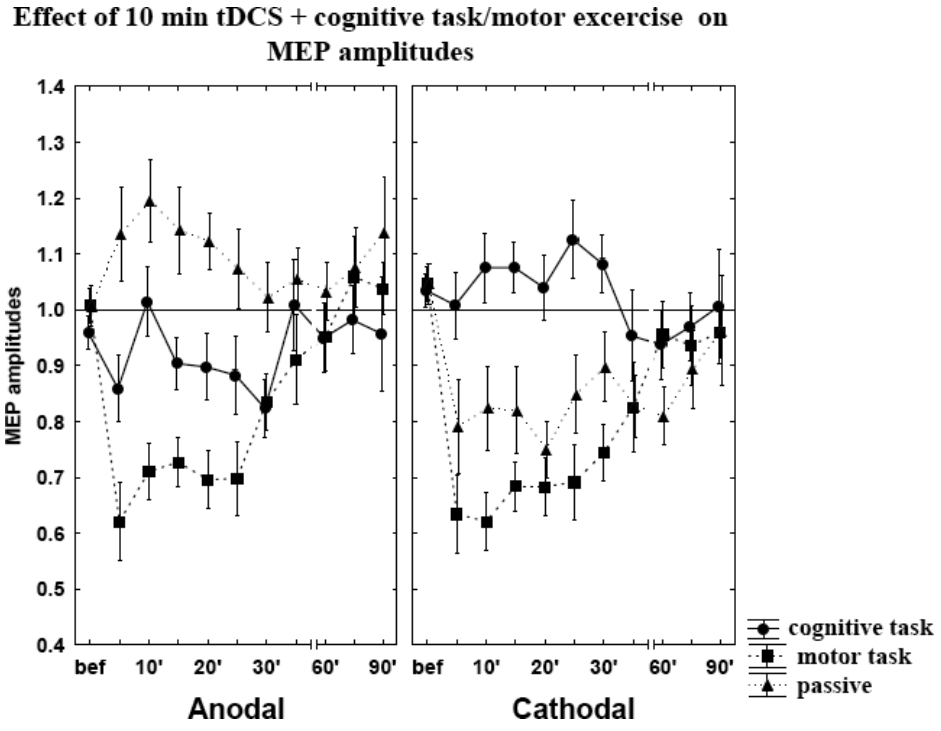

FIG. 1. Effect of 10 min anodal and cathodal stimulation on motor evoked potential (MEP) amplitudes. During the stimulation, the subjects were in a passive state (sitting), were required to complete a cognitive test presented on a computer monitor or had to push a ball with their right hand. The figure shows mean amplitudes and their SEMs up to 30 min (including all subjects, $n = 12$) and between 45 and 90 min (including six subjects). tDCS, transcranial direct current stimulation.

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modulation of neuroplasticity. PAS refers to a paradigm consisting of slow-rate repetitive low-frequency median nerve stimulation combined with TMS over the contralateral M1. Its principles of design were shaped after associative LTP in experimental animals, a mechanism likely to be relevant for learning and memory (for a review see Classen *et al.*, 2004). In the study by Stefan *et al.* (2004) PAS-induced plasticity was maximal when the subjects viewed their hand during stimulation and was reduced when the subjects only felt their hand. PAS failed to induce plasticity when the attention was directed towards the non-target hand or when a cognitive task was presented during stimulation.

The reduction of tDCS-induced plasticity during the cognitive task can be explained by previous neurophysiological (Motter, 1993) and imaging (Corbetta *et al.*, 1990; Rowe *et al.*, 2002) studies. These studies imply that cortical areas that are not involved in the processing of an attended task are deactivated. The processes of deactivation probably interfered with the neurophysiological processes (e.g. cortical inhibition) underlying tDCS-induced neuroplasticity. It should also be considered that tDCS was applied subthreshold, similarly to many previous studies, and higher current densities might result in different outcomes.

After the motor task a decline in the MEP amplitudes was observed after both types of stimulation, possibly due to the exercise and related muscle fatigue. Brasil-Neto *et al.* (1993) first observed that post-exercise MEPs were decreased when compared with pre-exercise MEPs, after an exhausting forearm exercise. Later studies have supported this result and additionally showed that post-exercise MEP reduction is often preceded by a short initial increase in MEP amplitude, called post-exercise facilitation, probably mirroring the neurotransmitter mobilization and depletion (Brasil-Neto *et al.*, 1994; McKay *et al.*, 1995; Liepert *et al.*, 1996). In our study we observed only a decrease after exercise and stimulation; however, we requested that the subjects have a 5 min break between the end of stimulation and the first recording session, in order to avoid spontaneous muscle contractions immediately after the termination of the voluntary contractions. As we did not observe any significant difference between cathodal and anodal stimulation, we suppose that the stimulation had no effect during this condition. However, a recent study has reported that anodal tDCS over the right motor areas resulted in an improved endurance time for a submaximal isometric contraction of the left elbow flexors, whereas the cathodal or no-stimulation condition did not produce such an effect (Cogiamanian *et al.*, 2007). In the same study it was also observed that, after the end of anodal stimulation, the amplitude of MEPs during a slight isometric biceps brachialis contraction (about 5% of the maximal voluntary contraction) increased significantly compared with the before-stimulation values. According to these results, anodal stimulation is able to modify human neuromuscular fatigue. However, in our study it is difficult to distinguish the reduction of cortical excitability due to muscle exercise from the effect of stimulation as we employed no sham condition. Furthermore, in the present study tDCS was applied during motor exercise (not during rest) and a different electrode position was used (left M1, right orbit vs. right M1, right shoulder). These technical differences might give rise to different results. Nevertheless, the purpose of our study was to investigate the effect of motor exercise on tDCS-induced neuroplasticity and not the effect of tDCS on fatigue.

In our study both the cognitive and motor tasks interacted with the tDCS protocol. The effect of tDCS is intracortical (Nitsche *et al.*, 2005). Whereas the effects during stimulation were probably due to the direct-current-induced shifts of resting membrane potential, the induction of longer lasting after-effects could well differ from these. Nevertheless, recent pharmacological studies proved that the after-effects of tDCS are NMDA receptor dependent (Liebetanz *et al.*, 2002). It is known that long-lasting NMDA-receptor-dependent cortical excitability and activity shifts are involved in neuroplastic modification. NMDA receptor and intracellular sigma 1 receptor blocker dextromethorphan intake prevented both anodal and cathodal tDCS-induced after-effects, demonstrating that dextromethorphan critically interferes with the functionality of tDCS irrespective of the polarity of direct current stimulation (Liebetanz *et al.*, 2002; Nitsche *et al.*, 2004b). D-cycloserine, a partial NMDA agonist, selectively potentiated the duration of motor cortical excitability enhancements induced by anodal tDCS (Nitsche *et al.*, 2004b). Additional receptors are also involved. Administration of the GABA(A) receptor agonist lorazepam resulted in a delayed but then enhanced and prolonged anodal tDCS-induced excitability elevation (Nitsche *et al.*, 2004a). In addition, dopaminergic mechanisms can stabilize these processes. In a recent study, the enhancement of D2, and to a lesser degree of D1, receptors by pergolide consolidated tDCS-generated excitability diminution up until the morning post-stimulation (Nitsche *et al.*, 2006).

In the present study we have proven that the effectiveness of tDCS in inducing motor cortical excitability changes depends on the cognitive state of the subjects and the activity level of the examined muscle induced by motor contraction. The limitation of our investigation is that results from a study employing healthy subjects cannot be directly transferable to clinical settings. Nevertheless, attentional and cognitive problems occur in older individuals and patients with varying neurological disorders more frequently than healthy subjects (Sprengelmeyer *et al.*, 1995; Claus & Mohr, 1996; Adler, 2005; Braak *et al.*, 2005; Finke *et al.*, 2006). Similarly, involuntary motor contractions and tremor are frequent symptoms of many neurological and psychiatric disorders (Marsden *et al.*, 1983; Benecke *et al.*, 1987; Rondot, 1991). The question that emerges is whether tDCS can be targeted accurately enough concerning the stimulation parameters to achieve a neuroplastic effect in these disorders.

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Abbreviations

FDI, first dorsal interosseus muscle; MEP, motor evoked potential; NMDA, *N*-methyl-D-aspartate; PAS, paired associative stimulation; tDCS, transcranial direct current stimulation; TMS, transcranial magnetic stimulation.

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Original Article

Pergolide Increases the Efficacy of Cathodal Direct Current Stimulation to Reduce the Amplitude of Laser-Evoked Potentials in Humans

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Abstract

Transcranial direct current stimulation (tDCS) was recently reintroduced as a tool for inducing relatively long-lasting changes in cortical excitability in focal brain regions. Anodal stimulation over the primary motor cortex enhances cortical excitability, whereas cathodal stimulation decreases it. Prior studies have shown that enhancement of D2 receptor activity by pergolide consolidates tDCS-generated excitability diminution for up to 24 hours and that cathodal stimulation of the primary motor cortex diminishes experimentally induced pain sensation and reduces the N2—P2 amplitude of laser-evoked potentials immediately poststimulation. In the present study, we investigated the effect of pergolide and cathodal tDCS over the primary motor cortex on laser-evoked potentials and acute pain perception induced with a Tm:YAG laser in a double-blind, randomized, placebo-controlled, cross-over study. The amplitude changes of laser-evoked potentials and subjective pain rating scores of 12 healthy subjects were analyzed prior to and following 15 minutes cathodal tDCS combined with pergolide or placebo intake at five different time points. Our results indicate that the amplitude of the N2 component was significantly reduced following cathodal tDCS for up to two hours. Additionally, pergolide prolonged the effect of the cathodal tDCS for up to 24 hours, and a significantly lowered pain sensation was observed for up to 40 minutes. Our study is a further step toward clinical application of cathodal tDCS over the primary motor cortex using pharmacological intervention to prolong the excitability-diminishing effect on pain perception for up to 24 hours poststimulation. Furthermore, it demonstrates the potential for repetitive daily stimulation therapy for pain patients. J Pain Symptom Manage

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Key Words
Pain, tDCS, pergolide, LEP, motor cortex, human

Introduction

In recent years, invasive stimulation of the primary motor cortex (M1) for the treatment of certain kinds of pain has attracted much interest. The first widely accepted clinical method for alleviating pain using cortical stimulation was epidural electrical motor cortex stimulation.[1,2] Recently, the most frequently investigated noninvasive method so far is repetitive transcranial magnetic stimulation (rTMS). Studies of transcranial magnetic stimulation in experimental and acute pain sensations have produced encouraging outcomes.[3,4] In spite of the beneficial effects of rTMS, a new method, transcranial direct current stimulation (tDCS) has been favored in recent editorials.[5,6]

Major advantages of tDCS as a tool for inducing long-lasting changes of cortical excitability and activity in focal brain regions is that it acts reversibly, painlessly, and safely.[7—10] Primarily, it causes polarity-dependent shifts of the resting membrane potentials and consequently changes the firing rates of neurons under the electrodes, neuronal projections and subsequent connected cortical areas.[11—13] Generally, anodal stimulation over the M1 has been found to enhance cortical excitability, whereas cathodal stimulation decreases it.[7,8] Although in humans the modulatory effect of tDCS had first been demonstrated on the motor system, it influences visual, somatosensory and prefrontal functions as well.[14—16] In a recent study, it was shown that enhancement of D2, and to a much lesser degree, of D1 receptor activity by pergolide consolidated cathodal tDCS-generated excitability diminution for up to 24 hours.[17]

Our first sham-controlled studies demonstrated that cathodal stimulation of the M1 diminishes experimentally induced pain sensation, and in parallel reduces the N2—P2 amplitude of laser-evoked potentials (LEPs) immediately after the end of stimulation.[18] The aim of the present study was to investigate the effect of combined cathodal stimulation and pergolide treatment on LEPs and related pain perception in a double-blind, randomized, placebo-controlled, cross-over study, with the clear intention of proving the already known inhibitory prolonging effect of pergolide[17] on pain perception. Here, amplitude changes of the N1, N2, and P2 of LEPs and subjective pain rating scores of 12 healthy subjects were analyzed prior to and following 15 minutes of cathodal tDCS, and following pergolide or placebo treatment at five different time points (before, 0 min, 40 min, two hours, 24 hours).

Methods

Subjects

Fifteen healthy volunteers (aged between 20 and 31 years) were informed about all aspects of the experiments and all gave an informed consent. Two participants chose not to continue the experiment after the first trials, and one subject was excluded as LEPs could not be identified reliably; 12 of the subjects (five male, seven female) were included in the study. All of the subjects underwent pergolide and placebo medication treatment. Additionally, seven subjects (three male, four female) participated in a control session in which no tDCS and drug treatment were introduced. We conform to the Declaration of Helsinki and the experimental protocol was approved by the Ethics Committee of the University of Göttingen. None of the subjects suffered from any neurological and psychological disorders, and none had metallic implants/implanted electric devices, nor took any medication regularly.

Pharmacological Interventions

Pergolide 0.025 mg combined with 10 mg domperidone to avoid side effects (e.g., vomiting induced by the medication) or equivalent

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placebo (glucose) was taken by the subjects orally two hours prior to the start of the experiments. By this means, the drug induces a stable plasma level[19] and produces prominent effects in the central nervous system.[17,20,21] To avoid cumulative drug effects, each experimental session was separated by at least one week. Subjects and the investigator conducting the experiment were blinded as to the respective pharmacological condition.

tDCS

tDCS was delivered by a battery-driven constant current stimulator (Eldith NeuroConn GmbH, Ilmenau, Germany) using a pair of rubber electrodes in a 5×7 cm water-soaked synthetic sponge. The cathode was placed over the representational field of the right abductor digiti minimi as identified by transcranial magnetic stimulation (Dantec S. A., Skovlunde, Denmark), whereas the other electrode (reference) was situated contralaterally above the right eyebrow. The electrodes were oriented approximately parallel to the central sulcus and the eyebrow. This montage has been proven to be the most effective for modulating motor cortex excitability.[7] The cathodal stimulation refers to the polarity of the electrode above the M1. The current was applied for 15 minutes with an intensity of 1.0 mA.

Laser Stimulation

A Tm:YAG laser system (WaveLight Laser Technologie AG, Erlangen, Germany) was used for the pain stimulation. The thulium laser emits near-infrared radiation (wavelength 2,000 nm, pulse duration 1 millisecond, laser beam diameter 7 mm) with a penetration depth of 360 μm into the human skin and allows a precise restriction of the emitted heat energy to the termination area of primary nociceptive afferents without affecting the subcutaneous tissue[22,23] The distal handpiece of the laser was positioned 30 cm from the radial part of the dorsal surface of the hand. Skin temperature of the stimulated area was checked prior to every switch between hands, and corrected with a heating lamp if it fell below 35°C. We stimulated slightly different spots in a square (5×5 cm) for each measurement to reduce receptor fatigue or sensitization by skin overheating.[23] In both experiments, the right hand was stimulated first in half of the cases and the left hand was stimulated first in the other half. This approach used as increased response toward novel stimuli, has been described in evoked potential studies of other sensory modalities.[24–27]

At the beginning of each condition the pain threshold of both hands was determined by applying laser stimuli from 200 mJ in 50 mJ steps. During EEG recording we delivered 40 laser pulses to each hand before and after tDCS with 1.5–1.6 times of threshold intensity. The interstimulus interval of the stimulation ranged from eight to 15 seconds. During each condition the intensity of the laser stimulation was kept constant as determined prior to tDCS enabling a clear comparison between results.

Psychophysical Evaluation

We used the numeric analog score to assess the subjective intensity of pain. The subjects were instructed to pay attention to the laser stimuli and to rate the perceived pain verbally (warm: 1, painful: from 2 (smallest) to 10 (most intensive pain)) about 2–3 seconds after each stimulation.[28] The ears of the subjects were plugged during the measurements to avoid auditory artifacts produced by the laser stimulation.

Electrophysiological Recordings

The electroencephalogram was recorded using a five-channel montage as described by Treede et al.[23] This montage has been used in numerous experimental and clinical LEP studies as it enables the easy identification of late LEP components. Electroencephalogram was recorded with gold disc electrodes from the Fz, Cz, Pz, T3, T4 (vs. linked mastoids) according to the international 10/20 system. The ground electrode was positioned on the forehead. The impedance was kept below 5 kOhm. Data were collected with a sampling rate of 1,000 Hz by the BrainAmp system (Brain Products GmbH, Munich, Germany) and were analyzed offline. A 0.5 Hz low cutoff and a 30 Hz high cutoff filter were used. After semiautomatic artifact detection (150-μV amplitude criterion), all epochs were visually inspected as well, and those containing eye blinks or muscle movement artifacts were excluded. All recordings consisted of at least 35

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artifact-free epochs. Baseline correction was performed on the basis of the 100-millisecond prestimulus interval. Using semiautomatic peak detection, we investigated different LEP components. The earliest component is a negativity N1 (peaking around 140−170 milliseconds), using T3 and T4 channels vs. Fz. The N1 component is followed by the late N2−P2 complex (N2: peaking around 160−220 milliseconds, P2: peaking around 300−360 milliseconds) in the midline (Fz, Cz, Pz) leads, using linked mastoid reference.

Experimental Procedures

The experiments were conducted in a repeated measurement design using a randomized order, with a break of at least one week between each session. Pergolide 0.025 mg or equivalent placebo (glucose) was taken by the subjects orally two hours before the start of the experiments. The subjects were seated in a reclining chair. First, the left motor-cortical representational field of the right abductor digiti minimi was identified using transcranial magnetic stimulation. At the beginning of each condition the pain threshold of both hands was determined by applying laser stimuli from 200 mJ in 50 mJ steps. During electroencephalogram recording we delivered 40 laser pulses to each hand before tDCS with 1.5−1.6 times of threshold intensity. Afterward, cathodal tDCS was performed for 15 minutes, followed by 40 laser pulses to each hand immediately after the stimulation, 40 minutes, two hours, and 24 hours later (Fig. 1).

Because our previous study has shown that sham stimulation has no significant effect on pain sensation,[18] no sham stimulation was used as an additional condition. However, we aimed to examine the normal habituation process. Seven subjects, chosen among the ones participating in the previous experiment, underwent the same protocol described previously, in which no tDCS and drug condition were introduced.

Data Analysis

Because the size of the amplitudes differed across subjects, normalization of the data was necessary. We divided the "after" tDCS-conditions by the value of the "before" condition. As a consequence of the bilateral representation of pain[29,30] and the lack of significant differences ($P > 0.05$) between the LEP amplitudes of the two sides, the data were not analyzed separately. Averaged numeric analog score values for N1, N2, and P2 amplitudes from each set of 40 trials were individually averaged and entered into a repeated-measures analysis of variance (ANOVA) (2 medications CONDITION [pergolide, placebo] × 4 TIME [after 0 min/before, after 40 min/before, after 2 hours/before, after 24 hours/before]). To compare the control results with the under medication conditions, all results of the seven subjects (3 CONDITION [pergolide, placebo, control] × 4 TIME) were also individually averaged and entered into a repeated-measures ANOVA. In addition, a one-way ANOVA was performed separately for each condition to show the effectiveness of direct current (DC) stimulation. We considered a psychophysical or an electrophysiological change if the CONDITION × TIME interaction was significant or if the one-way ANOVA revealed significant difference between time points. Post-hoc analysis was done using a Fischer LSD test.

Results

Psychophysics

The intensity of the laser stimulation was 21.32 mJ/mm^2 for pergolide medication (range, 19.5−23.4 mJ/mm^2), 21.177 mJ/mm^2 for placebo medication (range, 19.5−23.4 mJ/mm^2), and 21.32 mJ/mm^2 (range, 19.5−22.1 mJ/mm^2) for the control measurements (without tDCS and medication).

Concerning the pain perception scale, the ANOVA revealed no main effect of CONDITION [$F(1,23) = 2.38$, $P = 0.136$], but the effect of TIME was significant [$F(3,69) = 10.89$, $P < 0.005$]. The CONDITION × TIME interaction was not significant [$F(3,69) = 0.50$, $P = 0.680$]. If we compared the control results with the medication conditions, there was no main effect of CONDITION [$F(2,26) = 1.56$, $P = 0.229$], but the effect of TIME [$F(3,39) = 7.47$, $P < 0.005$] was significant (Fig. 2). There was no significant difference in the CONDITION × TIME interaction [$F(6,78) = 0.80$, $P = 0.570$].

One-way ANOVA revealed significant effect of TIME in the case of pergolide medication [$F(4,92) = 6.06$, $P < 0.005$]. The post-hoc analysis showed a significant difference between the before and after conditions ($P < 0.05$). In the

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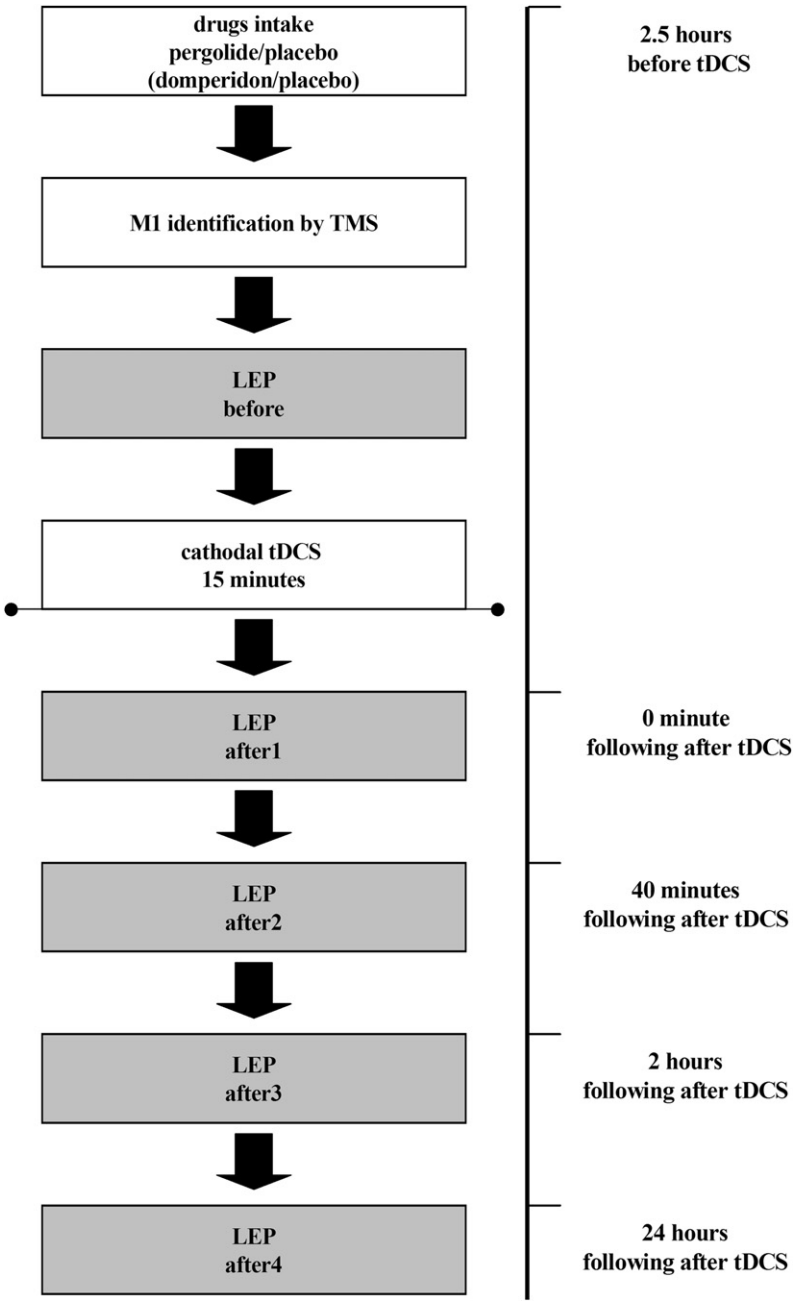

Fig. 1. Experimental procedure: Pergolide or an equivalent placebo drug was taken by all subjects orally two hours before the start of the experiments (2.5 hours before tDCS). First, the left motor-cortical representational field of the right abductor digiti minimi was identified using of TMS. During electroencephalogram recording we delivered 40 laser pulses to each hand before tDCS. Afterward, cathodal tDCS was performed for 15 minutes, following by 40 laser pulses to each hand immediately after the stimulation, 40 minutes, two hours, and 24 hours later.

case of placebo medication, the effect of TIME was significant [$F(4,92) = 4.54$, $P = 0.002$] and a significant difference was revealed between the after and all the other time points ($P < 0.05$). In the case of the control experiment, one-way ANOVA revealed no significant effect of TIME [$F(4,52) = 0.94$, $P = 0.446$].

The means and standard deviations of numeric analog score values for both hands and for all conditions are shown in Table 1.

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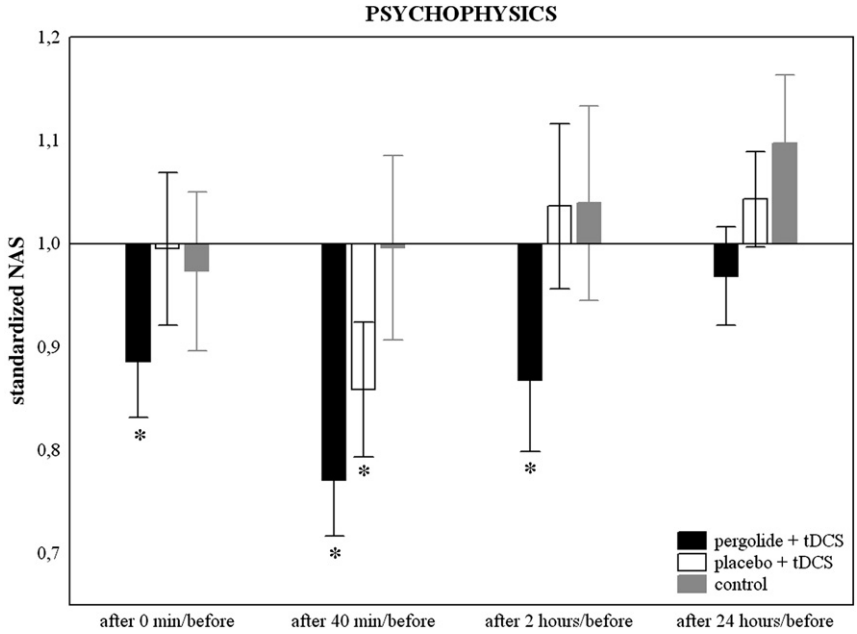

Fig. 2. The differences between numeric analog score results at four time points (standardized data by calculating the after 1−4/before ratio), for the two medication conditions (pergolide, placebo) and control experiment for both hands with laser stimulation. The standardized numeric analog score results show either an increase in pain sensation or a decline, relative to one. Following cathodal tDCS the pain sensation was lowered up to 40 minutes. The asterisks indicate significant differences between the different time points.

Electrophysiology

The laser stimulation induced a pricking pain and a biphasic N2−P2 component was clearly identified in all LEP measures of all 12 subjects (Fig. 3).

N1 Component. In the case of the N1 component, there was no main effect of CONDITION [$F(1,23) = 0.437$, $P = 0.515$] or TIME [$F(3,69) = 0.14$, $P = 0.937$] at the T3 electrode position. There was no significant CONDITION × TIME interaction [$F(3,69) = 0.28$, $P = 0.840$]. At the T4 electrode position, there was no main effect of CONDITION [$F(1,23) = 0.116$, $P = 0.325$] or TIME [$F(3,69) = 0.24$, $P = 0.654$]. There was no significant CONDITION × TIME interaction [$F(3,69) = 0.38$, $P = 0.213$]. In the control experiment, there was no main effect of CONDITION, or TIME. There was no significant difference in the CONDITION × TIME interaction ($P > 0.005$).

N2 Component. In the case of the N2 component, there was no main effect of CONDITION [$F(1,23) = 0.12$, $P = 0.737$], but the effect of TIME was significant [$F(3,69) = 7.44$, $P < 0.005$] at the Fz electrode position. There was no significant difference in the CONDITION × TIME interaction [$F(3,69) = 1.369$, $P = 0.259$]. At the Cz electrode position, there was no main effect of CONDITION [$F(1,23) = 0.91$, $P = 0.349$], but the effect of TIME was significant

Table 1
Averaged Numeric Analog Score Values and Standard Deviations from Each Set of 40 Trials

Condition	Side	Before	After1	After2	After3	After4
Pergolide + tDCS, $n = 12$	Left	4.91 ± 2.02	4.37 ± 1.98	4.06 ± 2.04	4.25 ± 2.27	4.89 ± 2.09
	Right	5.05 ± 1.77	4.30 ± 2.03	3.66 ± 2.09	4.50 ± 2.32	4.65 ± 1.79
Placebo + tDCS, $n = 12$	Left	4.05 ± 1.34	4.14 ± 1.41	3.55 ± 1.37	4.14 ± 1.72	4.36 ± 1.48
	Right	4.39 ± 1.39	3.98 ± 1.66	3.46 ± 1.34	4.41 ± 1.91	4.27 ± 1.32
Control, $n = 7$	Left	3.69 ± 1.76	3.31 ± 1.56	3.43 ± 2.15	3.61 ± 1.79	4.02 ± 1.40
	Right	3.28 ± 1.15	3.19 ± 1.32	3.59 ± 1.88	3.35 ± 1.90	3.49 ± 1.55

Averaged numeric analog score values from each set of 40 trials for the left and right sides separately and all conditions before and after tDCS.

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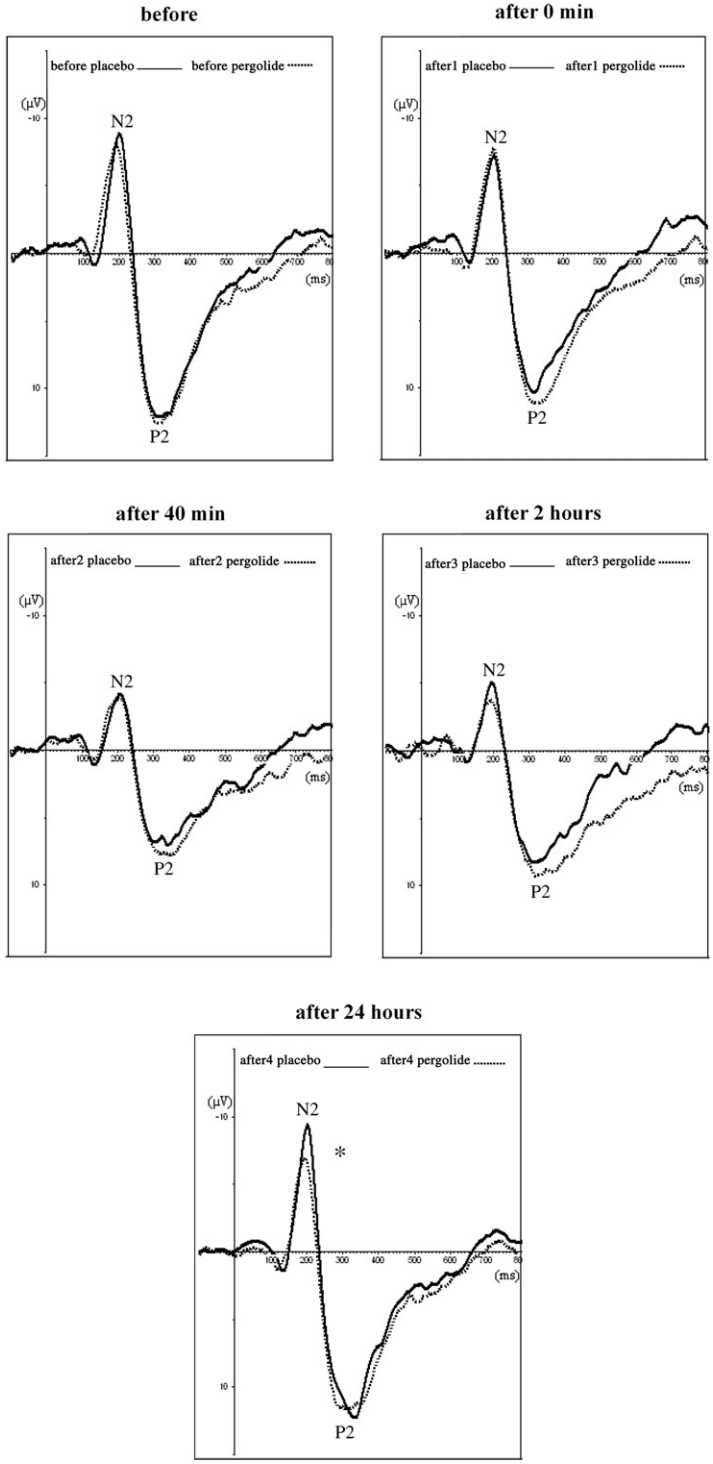

Fig. 3. Grand averages of LEPs obtained by both hand laser stimulation for the Cz recording electrode. The solid line shows LEPs for placebo medication combined with tDCS and the intermittent line for pergolide medication combined with tDCS at five different time points (before, after tDCS 0 min, after tDCS 40 min, after tDCS two hours, after tDCS 24 hours). Note that 24 hours following tDCS a greater amplitude reduction of the N2 component and N2P2 peak-to-peak amplitude for pergolide medication is observed when compared to placebo medication. The asterisk indicates a significant difference between the pergolide and placebo conditions.

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[$F(3,69) = 5.65$, $P = 0.001$]. We also found a significant CONDITION × TIME interaction [$F(3,69) = 3.67$, $P = 0.016$]. According to the post hoc analysis, pergolide medication, significantly decreased the amplitudes of N2 component, compared to the placebo medication, at the 24-hour time point ($P = 0.006$). However, there were no significant difference between the other time points when compared with the pergolide and placebo medication ($P > 0.05$). At the Pz electrode position there was no main effect of CONDITION [$F(1,23) = 1.93$, $P = 0.178$], but the effect of TIME was significant [$F(3,69) = 6.82$, $P < 0.005$]. There was no significant CONDITION × TIME interaction [$F(3,69) = 2.16$, $P = 0.101$].

To compare the control results to the under medication conditions, the results of the seven subjects were also individually averaged and entered into a repeated-measures ANOVA for all electrode positions. Although the effect of TIME was significant at all electrode positions ($P < 0.05$), neither the effect of CONDITION nor the CONDITION × TIME interaction were significant ($P > 0.05$).

One-way ANOVA revealed significant effect of TIME in the case of pergolide medication [$F(3,69) = 3.65$, $P = 0.017$]. The post hoc analysis showed a significant difference between the before and after conditions ($P = 0.002$). In the case of placebo medication, the effect of TIME was significant [$F(3,69) = 6.60$, $P < 0.005$] and a significant difference was revealed between the before and after, and after and after, conditions ($P < 0.05$). In the case of the control experiment, one-way ANOVA revealed no significant effect of TIME [$F(3,39) = 1.57$, $P = 0.211$].

P2 Component. Although the effect of TIME was significant ($P < 0.05$) in the case of the P2 component, there was no main effect of CONDITION, nor a significant CONDITION × TIME interaction at all electrode positions ($P > 0.05$). If we compared the control results with the under medication conditions, the effect of TIME was significant at all electrode positions, but neither the effect of CONDITION, nor the CONDITION × TIME interaction were significant ($P > 0.05$).

The means and standard deviations for the right and left hand separately, and under all conditions, are shown in Tables 2 and 3.

Discussion

tDCS modifies the excitability of the stimulated cortical area in a polarity-dependent way[7,8] and simultaneously causes perceptual changes.[31,32] In the present study, we explored the effects of this noninvasive brain stimulation technique on subjective acute pain perception and its electrophysiological correlates. Our results confirm that 15 minutes of cathodal tDCS over the primary motor cortex significantly reduced the amplitude of the N2 component, and the changes of the electrophysiological parameter remained stable for up to two hours after stimulation when compared to the control experiment (Fig. 4). Furthermore, the subjective pain sensation was lowered after cathodal tDCS for up to 40 minutes (Fig. 2).

Recording LEPs is a widely accepted method for examining the neuronal correlates of pain perception temporally and spatially in human subjects.[22,23] The earliest cortical LEP component is a negativity (N1, peaking around 140−170 milliseconds). According to its scalp topography (maximum near T3 and T4), it is

Table 2
Mean Values and Standard Deviations of the LEP Parameters for the Right Hand

Condition	Component	Before tDCS	After1 tDCS (0 min)	After2 tDCS (40 min)	After3 tDCS (2 hours)	After4 tDCS (24 hours)
Pergolide + tDCS, $n = 12$	N2 (μV)	−11.42 ± 6.40	−10.09 ± 5.55	−7.23 ± 4.49	−7.32 ± 5.97	−9.33 ± 5.33
	P2 (μV)	14.87 ± 5.54	12.82 ± 6.12	10.9 ± 6.14	11.68 ± 7.58	13.55 ± 5.90
Placebo + tDCS, $n = 12$	N2 (μV)	−11.04 ± 5.82	−9.36 ± 6.59	−6.78 ± 4.23	−7.38 ± 3.66	−11.39 ± 5.32
	P2 (μV)	15.14 ± 6.96	12.27 ± 7.11	9.07 ± 5.44	11.53 ± 5.23	14.76 ± 5.49
Control, $n = 7$	N2 (μV)	−13.12 ± 7.52	−11.83 ± 7.92	−10.31 ± 6.44	−8.67 ± 5.42	−12.45 ± 7.75
	P2 (μV)	12.94 ± 3.51	10.06 ± 3.21	8.79 ± 5.25	9.57 ± 3.83	11.32 ± 2.05

The table shows the mean values and standard deviations of the LEP parameters for all conditions at the Cz electrode positions obtained from all subjects for right hand before and after cathodal tDCS.

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Table 3
Mean Values and Standard Deviations of the LEP Parameters for the Left Hand

Condition	Component	Before tDCS	After1 tDCS (0 min)	After2 tDCS (40 min)	After3 tDCS (2 hours)	After4 tDCS (24 hours)
Pergolide + tDCS, $n = 12$	N2 (μV)	-11.73 ± 4.66	-9.98 ± 5.52	-7.45 ± 5.71	-5.83 ± 7.01	-8.5 ± 5.19
	P2 (μV)	15.22 ± 6.27	12.48 ± 6.87	10.38 ± 6.40	10.75 ± 6.39	14.22 ± 7.03
Placebo + tDCS, $n = 12$	N2 (μV)	-11.57 ± 6.96	-9.16 ± 5.81	-5.56 ± 4.15	-6.37 ± 4.52	-11.52 ± 6.30
	P2 (μV)	15.55 ± 8.60	11.83 ± 6.61	9.41 ± 6.89	11.61 ± 8.02	15.58 ± 7.02
Control, $n = 7$	N2 (μV)	-13.22 ± 6.70	-10.68 ± 7.76	-8.48 ± 4.95	-9.15 ± 5.93	-11.89 ± 5.84
	P2 (μV)	11.95 ± 3.52	8.67 ± 3.91	9.46 ± 3.85	9.61 ± 4.46	12.45 ± 2.46

The table shows the mean values and standard deviations of the LEP parameters for all conditions at the Cz electrode positions obtained from all subjects for left hand before and after cathodal tDCS.

probably generated near the secondary somatosensory cortex in the fronto-parietal operculum.[23] We did not find any significant change concerning the N1 amplitudes. Probably, this area could not be stimulated directly or the intensity of stimulation used was not sufficient to reflect any significant change.

The N1 component is followed by the late negative–positive complex (N2–P2) that can be most accurately recorded in the midline (Fz, Cz, Pz) leads. According to source localizing studies, the N2 component (peaking around 160–220 milliseconds) is generated both bilaterally in the operculoinsular region and partly in the anterior cingulate cortex (ACC).[2] This component contributes to sensory-discriminatory aspects of pain. The P2 component (peaking around 300–360 milliseconds) arises mainly from the ACC and reflects endogenous, attentional-cognitive,[33] and affective factors.[23,34] The role of ACC in coding pain intensity is still under debate; however, there is increasing evidence to suggest that activity in some parts of the ACC

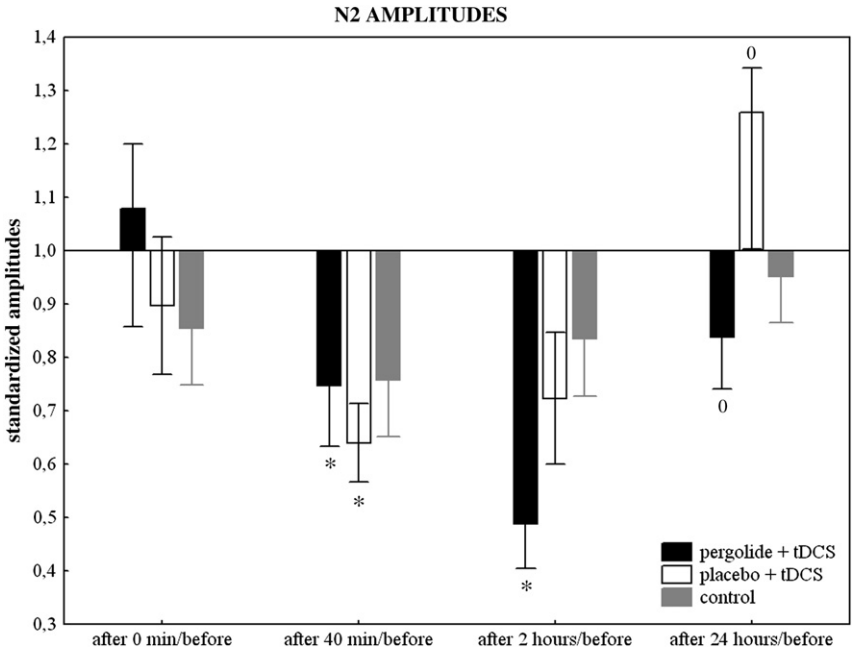

Fig. 4. The differences between mean N2 amplitude values at four time points (standardized data by calculating the after 1–4/before ratio), for the two medication conditions (pergolide, placebo) and control experiment for both hands laser stimulation at the Cz electrode. The standardized peak amplitudes show either an increase in the amplitude of the N2 component or a decline, relative to a value of one. Our results confirm that cathodal tDCS significantly reduced the amplitude of the N2 component when compared to the control experiment. The pergolide medication prolonged this effect for up to 24 hours. The asterisks indicate significant differences between the pergolide and placebo medications (**0**) or differences between time points (*).

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significantly correlates with increasing pain sensation.[35]

In a recent study, the effect of tDCS has been investigated by PET.[13] Concerning pain related regions, cathodal tDCS significantly diminished regional cerebral blood flow in the right ACC and the right thalamus. As the ACC is widely interconnected with primary and premotor areas,[36] it is likely that the stimulation of the M1 could result in a secondary inhibition of the ACC and as a consequence in N2 amplitude reduction. The antinociceptive effect as revealed by the psychophysical experiment could reflect the diminished involvement of the ACC in pain processing. DC stimulation of the left motor cortex resulted in several critical changes at the contralateral side.[13] Relative increases in regional cerebral blood flow after cathodal tDCS compared to sham tDCS were found in the right M1, frontal pole, primary sensorimotor cortex, and parietal occipital cortex. Regional CBF increase in homologous contralateral M1 was also found after rTMS to left M1.[37,38] Our results suggest that the stimulation of the left motor cortex has an influence on LEP components and pain perception of both sides. However, a recent study from Le Pera et al.[39] showed that the physiological activation of the motor cortex is able to reduce pain perception and LEP amplitude only when the motor area contralateral to painful stimuli is activated.

Relevant clinical studies show that repeatedly administering anodal tDCS over the M1 diminished pain sensation in patients with traumatic spinal cord injury[40] and induced significantly greater pain reduction compared with sham stimulation in patients with fibromyalgia.[41] However, comparing these results to our data, Fregni et al.[40,41] found pain reduction after anodal tDCS. The divergent results can be explained by the difference between acute and chronic pain processing. Pathological changes due to chronic pain are characterized by many functional and structural changes in the brain[42,43] and these cortical reorganizations probably lead to changes in cortical excitability.

In our study, the oral administration of pergolide prolonged the effect of cathodal tDCS for up to 24 hours on LEPs. The possible mechanisms of DC-induced after-effect were investigated by several previous studies. Pharmacological intervention suggests that the after-effect is *N*-methyl-D-aspartate (NMDA)-receptor dependent.[44–46] Dextromethorphan (NMDA-receptor and intracellular sigma 1 receptor blocker) intake prevented both anodal and cathodal tDCS-induced after-effects, demonstrating that dextromethorphan critically interferes with the functionality of tDCS, irrespective of the polarity of DC stimulation.[44] It is known that long-lasting NMDA-receptor dependent cortical excitability and activity shifts are involved in neuroplastic modification. Dopaminergic mechanisms stabilize these processes, as shown by animal experiments.[47–49] Dopamine (DA) resident in the synapses could strongly influence the induction of long-term potentiation and/or long-term depression through specific changes in the initial levels of cAMP and Ca^{2+}, which are key regulators of LTPs in the hippocampus, striatum, and prefrontal cortex.[50–53] One study showed that long-term potentiation dependent processes such as practice-dependent plasticity are enhanced by DA.[54] DA acting on D1 receptors increases NMDA currents.[55] In a recent study, the dopaminergic influence on NMDA receptor-dependent neuroplasticity was investigated using tDCS. The enhancement of D2, and to a lesser degree, of D1 receptors by pergolide consolidated tDCS-generated excitability diminution up until the morning poststimulation.[17] Our results are in agreement with this study.

An antinociceptive effect of pergolide is implausible in the case of a single oral dose of 0.025 mg. Pronociceptive or antinociceptive effects of pergolide have not yet been published. However, the administration of levodopa, an indirect DA agonist, has been reported to reduce pain ratings in painful diabetic neuropathy in humans.[56] The DA reuptake inhibitor bupropion also has analgesic effects.[57] Contrary to these results, the systematic administration of DA D2 receptor antagonist in humans has also been shown to reduce pain ratings in clinical trials.[58,59]

The reduction of pain perception and the amplitudes of N2 and P2 components could also be due to a normal habituation observed by several studies.[60,61] Therefore, we have repeated the measurements in the absence of tDCS and medication. Although, during the control experiment, a normal habituation process was observed (the N2 and P2 amplitudes

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were reduced insignificantly), our results demonstrated that medication conditions with tDCS induced a significant amplitude reduction of the N2 peak. In addition to this, the psychophysical evaluation did not reveal significant changes in subjective pain perception during the control experiment.

To summarize, we observed that following cathodal tDCS the N2 amplitude of the LEP components were significantly decreased when compared to the control experiment, and simultaneously, pain sensation was reduced. The changes in LEPs remained stable for up to two hours after 15 minutes of cathodal stimulation and pergolide prolonged the effect of the cathodal tDCS, causing a decrease in the amplitude of the N2 component for up to 24 hours. Our findings were based on experimentally induced pain using LEPs in a population of healthy subjects. The limitation of our investigation is that results from a study using healthy subjects cannot be directly transferable to clinical settings. However, on the way toward a clinical application of either rTMS or tDCS, to our knowledge, this is the first study observing plasticity-prolonging effects of drugs affecting the CNS on a clinically relevant behavioral range. Two principle effects may be relevant in further clinical studies: prolongation of excitability enhancing effects for diseases such as stroke and Parkinson's disease by, for example, amphetamine[62,63] or d-cycloserine;[64] and prolongation of inhibitory after-effects, as shown here, on pain, epilepsy, or other diseases associated with cortical hyperexcitability.

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