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.

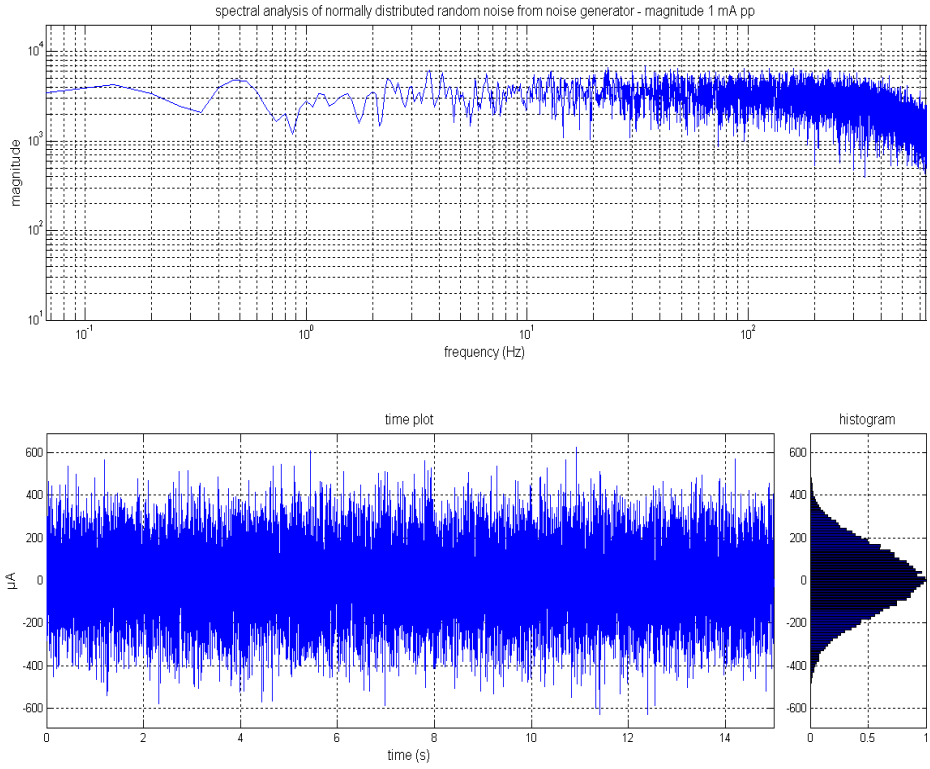
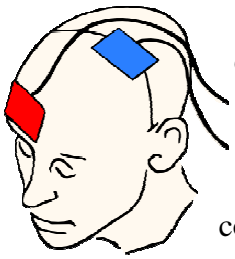


Figure 1. The figure shows the output signal of the DC-Stimulator PLUS, as a frequency distribution of the signal; the time plot of the signal and as a histogram. The signal was generated by a computer. In the stimulation mode "noise" there is a random level of current generated for every sample (sampling 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.

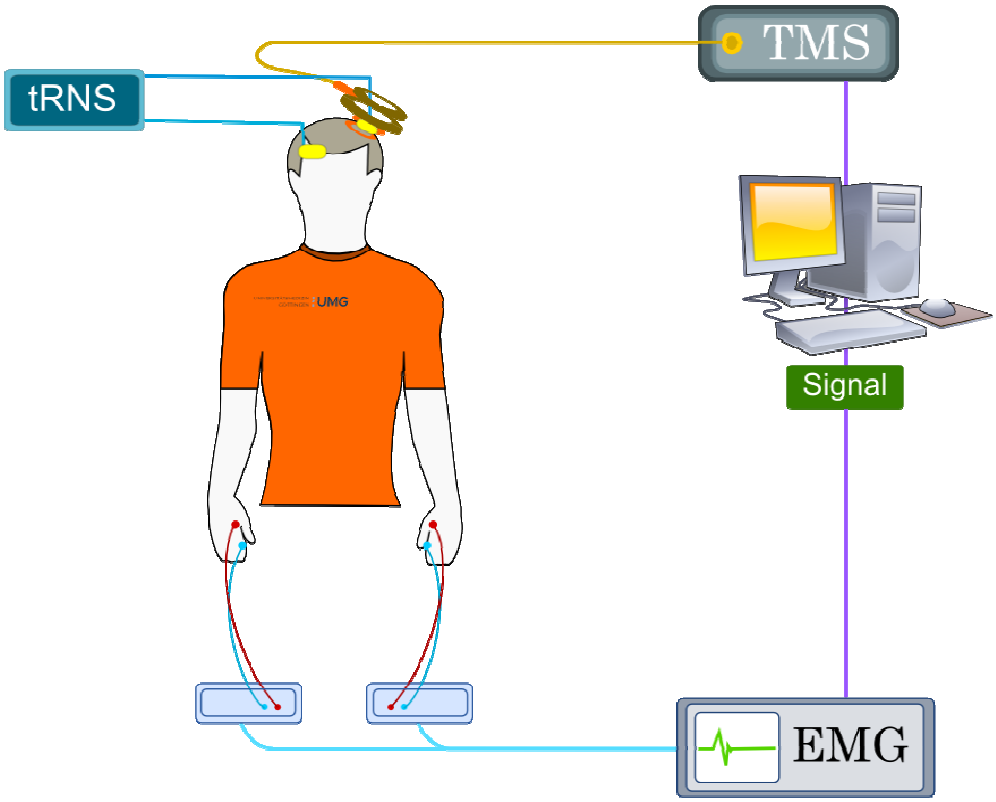


Figure 2. Methods and materials. MEPs of the right FDI muscle were recorded following stimulation of its motor-cortical representational field by single-pulse TMS. These were induced using a Magstim 200 magnetic stimulator, with a figure-of-eight standard double magnetic coil. Surface EMG was recorded 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).

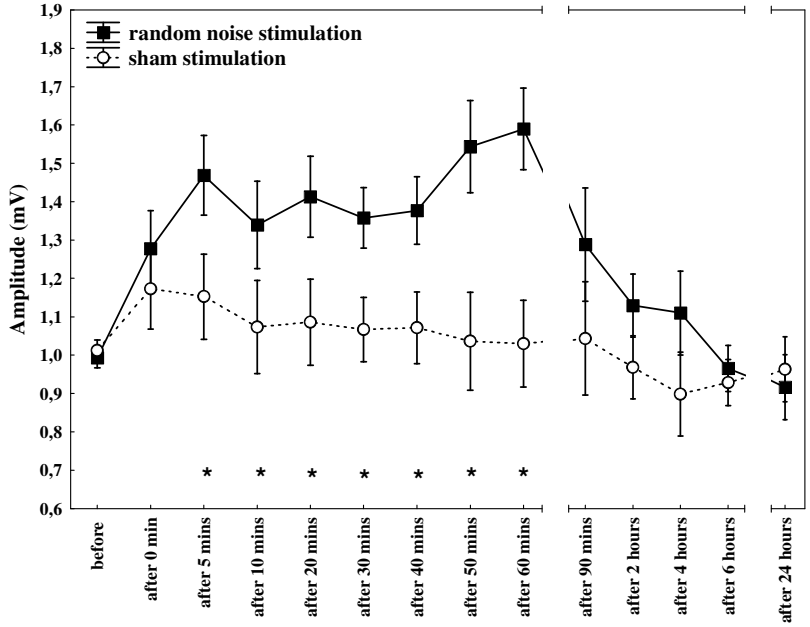


Figure 3. Effect of 10 min RN stimulation on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min RN stimulation over M1 at 1mA, compared to sham stimulation. The figure shows mean amplitudes and their SEMs up to 60 min (including 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.

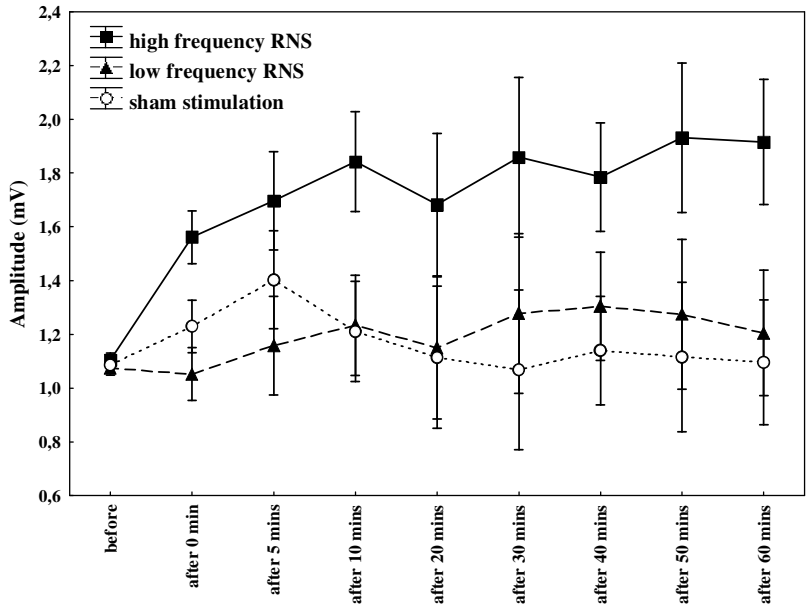


Figure 4. Effect of 10 min low- (0.1 Hz-100 Hz) and high-frequency (101 Hz-640 Hz) RN stimulation on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min high-frequency RN stimulation over M1 at 1mA, compared to low-frequency and 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.

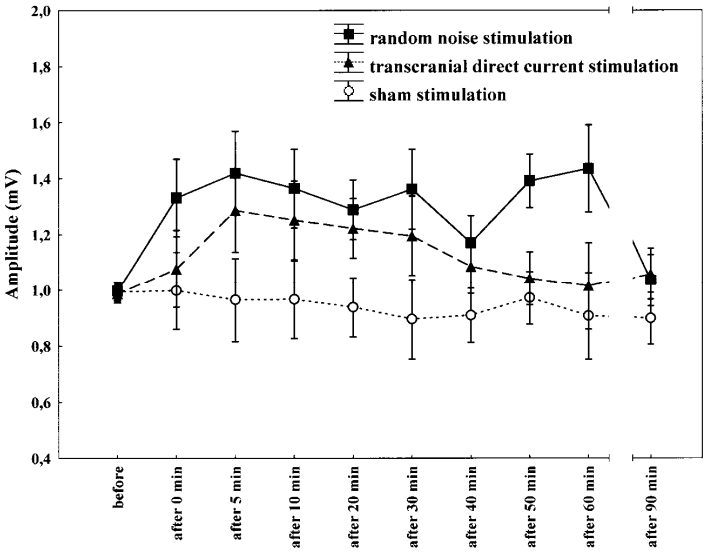


Figure 5. Effect of 10 min tRNS and anodal tDCS on motor evoked potentials. Time course of M1 excitability changes lasting for 60 minutes post-stimulation, shown after 10 min RN stimulation over M1. However, the facilitation of MEP size following anodal tDCS lasts for approximately 40 mins. The figure 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.

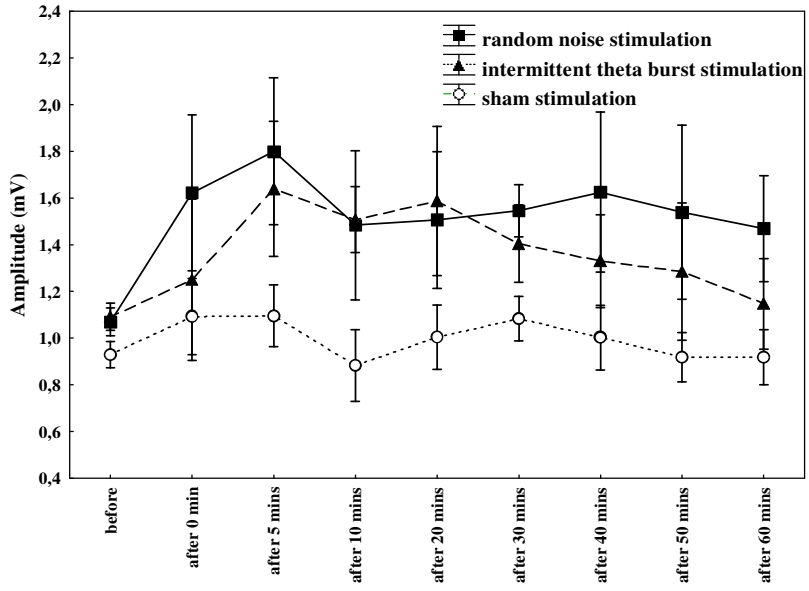


Figure 6. Effect of tRNS and rTMS on motor evoked potentials. The pattern of rTMS consisted of bursts containing 3 pulses at 50 Hz at an intensity of 80% AMT, repeated at 200 ms intervals (i.e., at 5 Hz). 2s train of TBS was repeated every 10s for a total of 190 s (600 pulses). The time course of M1 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.

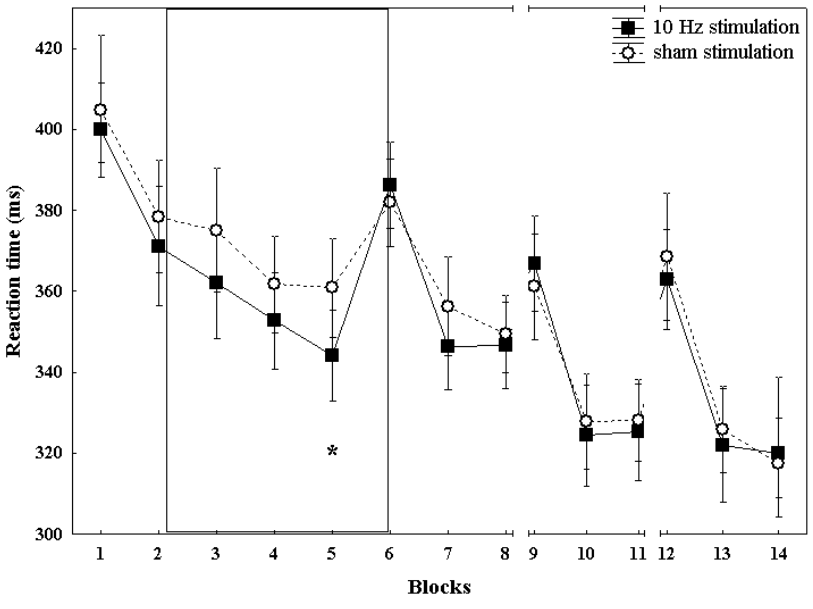
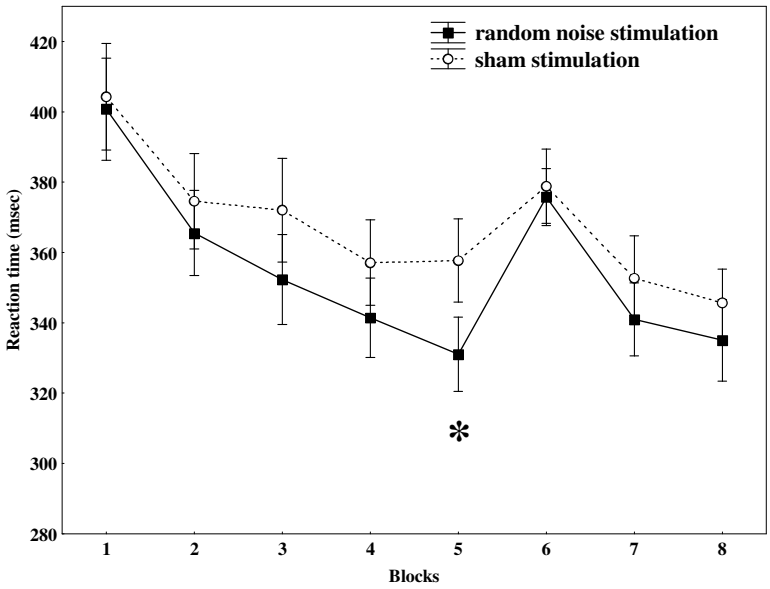
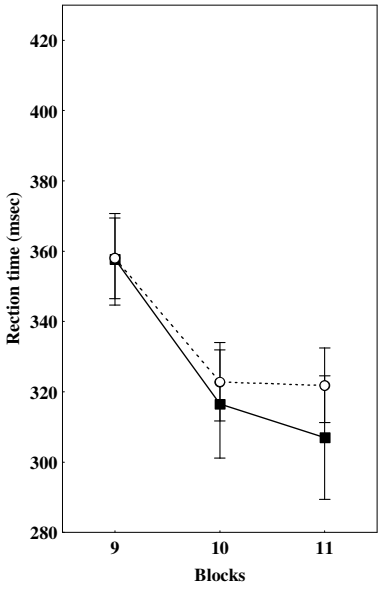


Figure 7. 10 Hz tACS of the M1 improves implicit motor learning in its early phase. Reaction times decrease faster in the 10 Hz stimulation condition compared to the sham stimulation condition. Moreover, the RT difference comparing blocks 5 and 6, which indicates implicit sequence learning most purely, 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).





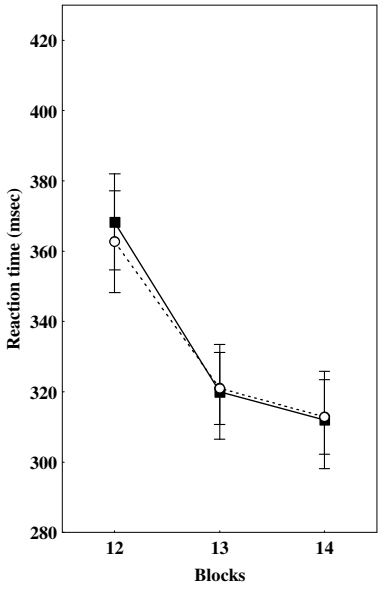


Figure 8. TRNS of the M1 improves implicit motor learning in its early phase. Reaction times decrease faster in the tRNS condition when compared to the sham stimulation condition (upper figure). Moreover, the RT difference comparing blocks 5 and 6, which indicates implicit sequence learning, is bigger 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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