Neuroplastic effects of transcranial near-infrared stimulation (tNIRS) on the motor cortex

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Running title: tNIRS modulates cortical excitability in the M1

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List of abbreviations:

AMT:	active motor thresholds
CCO:	cytochrome C oxidase
EMG:	electromyogram
FDI:	first dorsal interosseous muscle
ICF:	intracortical facilitation
ISI:	interstimulus intervals
M1:	primary motor cortex
MEPs:	motor evoked potentials
RMT:	resting motor thresholds
SICI:	short intracortical inhibition
SRTT:	serial reaction time task
tACS:	transcranial alternating current stimulation
tDCS:	transcranial direct current stimulation
tNIRS:	transcranial near-infrared stimulation
TMS:	transcranial magnetic stimulation
tRNS:	transcranial random noise stimulation

Abstract

Background: Near-infrared light stimulation of the brain has been claimed to improve deficits caused by traumatic brain injury and stroke.

Objective: Here, we exploit the effect of transcranial near-infrared stimulation (tNIRS) as a tool to modulate cortical excitability in the healthy human brain.

Method: tNIRS was applied at a wavelength of 810 nm for 10 minutes over the hand area of the primary motor cortex (M1). Both single-pulse and paired-pulse measures of transcranial magnetic stimulation (TMS) were used to assess levels of cortical excitability in the corticospinal pathway and intracortical circuits. The serial reaction time task (SRTT) was used to investigate the possible effect of tNIRS on implicit learning.

Results: By evaluating the mean amplitude of single-pulse TMS elicited motor-evoked-potentials (MEPs) a significant decrease of the amplitude was observed up to 30 minutes post-stimulation. The short interval cortical inhibition (SICI) was increased and facilitation (ICF) decreased significantly after the tNIRS. The results from the SRTT experiment show that there was no net effect of stimulation on the performance of the participants. Significant differences between female and male subjects were observed; female subjects being faster but less accurate during stimulation. Results of a study questionnaire demonstrated that tNIRS did not induce serious side effects apart from light headache and fatigue. Nevertheless, 66% were able to detect the difference between active and sham stimulation conditions.

Conclusion: tNIRS offers the potential to induce neuroplastic changes in the intact human cortex with a high spatial resolution.

Introduction

The primary target of applying infrared light as a therapeutic tool is for wound healing, inflammation and chronic pain relief. Applications have been widened to include the potential of rehabilitative treatment for neurological disorders which has been extensively investigated using animal models (1-3), in early clinical trials of patients with stroke and traumatic brain injury (4-8), as well as recently showing promise as a potential treatment for Alzheimer's disease (9).

The transcranial application of near infrared light (tNIRS) to tissues in both the peripheral and the central nervous system has been performed for at least a decade and stimulation parameters like wavelength, fluence, irradiance, treatment duration and timing, continuous or pulsed stream of laser light have been investigated (10-12). A U-shaped response curve characterizes the optimum dosage of laser light promoting wound healing and regeneration of tissue, while a higher dosage has a detrimental effect due to heating of the tissue (5, 12-14). TNIRS therapies, applied in optimized dosages have been claimed to produce remarkable and reproducible effects both in the brain and peripheral tissues after traumatic insult in both animal models of disease and in humans (6, 10, 15). The outcomes of these studies have led to the establishment of a multinational stroke trial currently in its third phase (NCT01120301) to investigate the application of tNIRS in stroke rehabilitation and its ability to limit cognitive deficits post stroke onset (4, 7, 8).

The putative mechanism of action of infrared light is believed potentiate the cytochrome C oxidase (CCO or complex IV) complex in the mitochondria, a component of the electron transport chain and key complex in ATP production. The action spectrum of CCO is in the near-infrared range. As tNIRS is applied at a wavelength of 810nm in the NIR range, this suggests that CCO might play a key role in the cellular response of the stimulation (16).

Here, we provide evidence that tNIRS is suitable as a tool for influencing cortical excitability and activity in the healthy human brain. We have applied tNIRS in an efficient dosage over the cortical representation of the hand area of the primary motor cortex (M1) using a constellation of four laser diodes attached to percutaneous acupuncture needles.

Materials and Methods

The study was approved by the ethics committee of the University of Göttingen and conformed to the Declaration of Helsinki. All participants were informed as to all aspects of the experiments and gave written consent.

Subjects

Altogether 56 right handed volunteers in the age range of 18-35 years were recruited, passed a standard physician's examination and met further inclusion criteria: no neurological or psychiatric disorders, pacemaker, metal implants in the head region, pregnancy, drug or alcohol addiction, or participation in another study within the last 6 weeks.

Transcranial near-infrared laser stimulation (tNIRS)

tNIRS was applied using stainless steel laser acupuncture diode needles which are sterilized after each use. Each diode is connected to an optic fiber cable and outputs 50mW per diode at a wavelength of 810nm at 35% of the NIR laser stimulator output. The laser needles were placed over the M1, at the 'hotspot' predetermined by TMS and held in place with wire holders attached to a crown that wraps around the head of the participant (Fig.1.). In a control condition including 8 subjects the visual cortex was stimulated. The laser stimulator

(WeberMedical, GmbH) was programmed to administer tNIRS for 10 mins; after this it shuts down stimulation automatically. Subjects had to participate in 2 experimental sessions, receiving either placebo or active stimulation. During the placebo condition the laser was switched on for a 30 sec period only. A minimum of 4 days were maintained between each session to avoid any carry-over effects of the stimulation.

Measurement of motor-cortical excitability

To detect changes in excitability motor-evoked-potentials (MEPs) of the right first dorsal interosseous (FDI) were recorded following tNIR stimulation of its motor-cortical representational field by single-pulse TMS. These were elicited using a Magstim 200 magnetic stimulator (Magstim, Whiteland, Dyfed, UK) and a figure-of-eight magnetic coil (diameter of one winding=70mm; peak magnetic field=2.2 Tesla). The coil was held tangentially to the skull, with the handle pointing backwards and laterally at 45° from the midline. The optimal position was defined as the site where stimulation resulted consistently in the largest MEP. Surface EMG was recorded from the right FDI with Ag–AgCl electrodes in a belly tendon montage. Raw signals were amplified, band-pass filtered (2 Hz-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 offline analysis. The intensity of the stimulator output was adjusted for baseline recording so that the average stimulus led to an MEP of 1 mV in amplitude.

Resting motor threshold (RMT), active motor threshold (AMT), the intensity required to elicit an MEP of ~ 1mV peak-to-peak amplitude (SI1mV) and a baseline of TMS-evoked MEPs at the defined SI1mV intensity, were recorded at 0.25 Hz prior to 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 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 MEPs (200 μV) in the tonically contracting FDI muscle (17).

Experimental procedures

15 subjects (7 male) participated in the single-pulse and in the intracortical excitability sections of the study. The experiments were conducted in a randomised, repeated measurement design. In order to exclude the unspecific effect of the stimulation, 8 naïve subjects (3 males) participated in a control study, in which the visual cortex was stimulated.

Recording of single-pulse MEPs: A baseline of TMS-evoked MEPs (30 stimuli) was recorded at 0.25 Hz. After termination of tNIRS, 30 MEPs were recorded at 0.25Hz 0mins, 5mins and then every 10 mins up to 60 mins poststimulation.

Measures of intracortical excitability: SICI/ICF and LICI were measured using two different protocols 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 (18). To avoid any floor or ceiling effect, the intensity of the conditioning stimulus was set to 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 with the test pulse alone was tested 40 times and each of the conditioning-test stimuli 20 times (the same paradigm was used for LICI). 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 was LICI which applies two suprathreshold stimuli with ISIs of 50, 100, 150, and 200 ms (19). The intensity of both stimuli was set to 110 % of RMT. LICI was taken as the mean percentage inhibition of the conditioned test pulse MEP at ISIs of 50, 100, 150, and 200 ms. Recruitment curves were measured using stimulus intensities of 110 %, 130 % and 150 % of RMT, each with a train of 10 pulses. Measurements were performed prior to active and sham stimulation sessions, immediately and 30 min poststimulation.

Serial reaction time task (SRTT): The SRTT (20) is an established test to investigate implicit motor learning also in the context of brain stimulation (Nitsche et al, 2003). During the task the participant has to respond to a visual cue as fast and as accurately as possible with individual finger movements in response to a four dot sequence on the computer screen. Participants are unaware that the sequences follow a pseudo-repeating pattern, but their ability to implicitly 'learn' the sequence is measured over the course of the task. The task is divided into 8 blocks. Blocks 1-5 and blocks 7 and 8 have the same pattern, whereas the sequence in block 6 is different to the other sequences presented in the other blocks. The calculated difference in the participants' reaction times in block 6 compared to their performance in block 7 is considered to be a measure of implicit motor-learning. Effects of transcranial stimulation using the SRTT have been shown to be a robust measure of this kind of learning and the structure of the paradigm ensures a specific sequence learning is measured and prevents an unspecific decreased reaction time purely due to increasing task routine (21).

32 subjects (16 male) participated in this part of the study. The subjects were seated in front of a computer screen placed at eye level and were not informed as to the aim of the SRTT. Their right fingers were placed on the computer keyboard on the designated keys for each finger. Four bars appeared on the screen: the first from the left corresponding to the right index finger, the second the middle finger, the third the ring finger and the fourth the little

finger. Ten minutes tNIRS or sham stimulation was given during the performance of the task. 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 in a given experimental condition, mean RT was calculated for each subject separately.

Questionnaires

To examine safety aspects and to evaluate the blinding efficacy of tNIRS participants were asked to fill out questionnaires examining the cutaneous effects of tNIRS; side effects like burning sensations, tingling, itching and pain, fatigue, nervousness and differences in concentration as well as any other noticeable sensations were documented. The questions concerned sensations during and after the stimulation. 28 questionnaires were filled out correctly (15 active and 13 sham sessions).

Data analyses

Single-pulse TMS. MEP amplitude means were calculated for each time point covering baseline (30 stimuli) and poststimulation time-points (30 stimuli). Baseline normalised MEPs were analysed using repeated measurements of ANOVA (CONDITION (tNIRS vs. sham) x TIME (0, 5, 10, 15; 20, 25; 30, 40, 50, 60 min post-stimulation). Effects were considered significant if $p<0.05$. In the case of a significant main effect or interaction, a Student's t-test was performed. Student's t-test was used to compare the MEP values between baseline and post-stimulation measurements within group. All data are given as means + SEM.

Paired-pulse TMS. For each measurement (SICI, LICI, recruitment curves (I/O)), 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 CONDITION (tNIRS vs. sham), the ANOVA model included the factor "ISI" (2, 4, 7, 9, 12) when SICI (50, 100, 150, 200) was analyzed or the factor "intensity" (100%, 130%, and 150% of RMT) for recruitment curves. A p value of <0.05 was considered significant for all statistical analyses. In the case of a significant main effect or interaction between ISI/intensity and stimulation condition, a t-test was performed.

SRTT analysis. A repetitive measures ANOVA (independent variables: CONDITION and BLOCK) for reaction time (RT) and error rate (ER) was performed. As the RT difference between Block 5 and 6 is thought to represent an exclusive measure of implicit learning interactive Students´ t-tests were performed to compare the respective differences between tNIRS and sham conditions. A p value of <0.05 was considered significant for all statistical analyses.

Results

All of the subjects tolerated the stimulation; none of the experimental sessions were interrupted or terminated due to side effects of the stimulation.

Single-pulse MEPs

RMT and AMT baseline values were compared between tNIRS and sham conditions using Student's t-test. There was no significant difference in any of the measurements ($p>0.4$).

After 10 min tNIRS cortical excitability decreased by 20 - 30%, as revealed by single-pulse TMS. Repeated measurements of ANOVA revealed a significant main effect of

CONDITION ($F(1,14)=10.21$, $p=0.006$). The main effect of TIME ($F(9,126)=1.33$, $p=0.23$) and the interaction between CONDITION and TIME were not significant ($F(9,126)=0.73$, $p=0.67$) (Figure 2A). According to the t-test, significantly decreased MEPs were observed at the 0 and 30 min compared to the baseline ($p<0.05$). Individual data can be seen on Figure 2BC. Gender differences were not observed ($p>0.6$).

The stimulation of the visual area did not result in any MEP change, compared to the sham condition (CONDITION: $F(1,7)=0.21$, $p=0.66$; TIME: $F(9,63)=0.73$, $p=0.68$; CONDITION x TIME: $F(9,63)=1.21$, $p=0.3$).

Paired-pulse TMS

With regard to SICI repeated measurements of ANOVA revealed a significant effect of ISI ($F(4,48)=63.81$, $p<0.001$) and CONDITION ($F(1,12)=7.99$, $p=0.015$), which was due to the significantly increased inhibition immediately at the end of the tNIRS at the ISI of 2 ms ($t=2.48$, $p=0.028$) and decreased excitation at the ISI of 9 ms ($t=3.58$, $p=0.0037$) (Figure 3). There were no other significant main or interaction effects with regard to SICI (Table 1.). Gender differences were not observed ($p>0.5$)

tNIRS had no significant effect on LICI and motor-evoked recruitment curves (Table 3) as revealed by repeated measurements of ANOVA (Table 2). Gender differences were not observed ($p>0.9$).

Implicit motor learning

Repeated measures ANOVA revealed a significant main effect on BLOCK ($F(7,217)=22.20$, $p<0.001$) There was no significant effect on CONDITION ($F(1,31)=0.2$, $p=0.66$) and the CONDITION x BLOCK interaction was also not significant ($F(7,217)=0.43$, $p=0.88$). Nevertheless, it was clearly seen that the RTs between the female and male subjects are different. Fig. 4 shows the raw RTs in the two groups before and during tNIRS. A significant CONDITION x GENDER interaction was observed ($F(1,30)=4.48$, $p=0.04$) and a marginally significant CONDITION x GENDER x TIME interaction ($F(7,210)=1.83$, $p=0.08$) was also detected, due to the significant RT decrease in the female group after Block 3 during active stimulation ($p \leq 0.05$) (Figure 4).

With regard to the ER, repeated measures ANOVA revealed a significant main effect on BLOCK ($F(7,217)=17.26$ $p<0.001$) There was no significant effect on CONDITION ($F(1,31)=0.13$, $p=0.72$) and the CONDITION x BLOCK interaction was also not significant ($F(7,217)=0.53$, $p=0.16$). A significant main effect of GENDER was observed ($F(1,30)=9.28$, $p=0.005$). Furthermore the CONDITION x GENDER interaction ($F(1,30)=4.7$, $p=0.038$) and GENDER x TIME interactions ($F(7,210)=2.26$, $p=0.03$) were significant, due to the higher ER rate in the female group (Figure 5). Student's t–test revealed no significant differences between active and sham stimulation conditions in the female and male groups, although at block 6 a tendency was observed in the female group ($p=0.09$). However, significant differences between males and females during sham stimulation at block 6 ($p=0.04$) and 8 ($p=0.01$) and during active stimulation at each block ($p<0.01$) were observed.

Perceptual sensations and side effects during and after stimulation

During active stimulation 100% of the subjects reported feeling burning sensation during active and 8% during sham stimulation, with a mean intensity of 3.5 (VAS=5). Pain

and tingling were reported by 60 and 47% of the subjects respectively. Fatigue was higher in the sham group (38% vs 27%) during stimulation (Table 4). 66% of the subjects were able to distinguish between sham and active stimulation with a high confidence. After active stimulation 26 % of the subjects experienced burning and pain sensations. Itching and tingling sensations were similar in both groups (between 8 and 16%). Light headache was reported by 20% of the participants.

There was no difference in the number of female and male subjects who reported experiencing tingling, burning and pain during stimulation, but their perception intensities varied. Female participants stated 1.5 times higher tingling, burning and pain sensations. However, fatigue, nervousness and itching were felt by male subjects twice as much than by females during stimulation. No gender specificity was observed after stimulation.

Discussion

Supporting previous findings (22) we have shown that a 10 min. application of tNIRS to the M1 can inhibit cortical excitability as measured by attenuation of the amplitude of TMS-elicited MEPs. Near-infrared light can penetrate the intact skull as demonstrated by many publications on near-infrared spectroscopy (NIRS). NIR enters through the skull further and reaches deeper tissue than red light (14). The duration of the induced inhibition was longer than the stimulation itself: the MEP amplitudes reached baseline values after 30 minutes poststimulation.

We have further shown that MEP inhibition was due to an increased SICI and a decreased SICF after active stimulation. tNIRS had no significant effect on LICI and motor-evoked recruitment curves. SICI reflects intracortical inhibition and is mediated by gamma aminobutyric acid ($GABA_A$) receptors, whereas ICF is most likely mediated by the

glutamatergic system (23). Therefore, it is possible that tNIRS both increases GABAergic neurotransmission and/or decreases glutamatergic actions.

Infrared light most likely acts at the level of the mitochondria in targeted cells (13). *In vitro* experiments have shown that laser irradiation modulates mitochondrial respiration levels, and is increased following irradiation of cellular tissues, causing an amplification of mitochondrial products, such as ATP, nicotinamide adenine dinucleotide (NADH), protein and ribonucleic acid (RNA) (24). In neuronal mitochondria, CCO is the most likely candidate target. tNIRS, therefore, could increase the process of cellular respiration in neurons (by either preferentially modulating the activity of neurons in inhibitory networks, or inhibitory neurons themselves, as assumed here), by increasing energy and cyclic adenosine monophosphate (cAMP) levels.

A second potential mechanism of how near-infrared light can affect neurons is through the dissociation of nitric oxide (NO) and oxygen (5). In a stressed or damaged cell, the mitochondrial NO production is upregulated. NO has a high affinity for binding to the CCO complex and under conditions of cellular stress NO binds to the same binding site as oxygen and prevents oxygen from binding to the CCO complex, which reduces cellular respiration and available ATP. By the action of laser induced NO dissociation from the CCO complex, the ongoing cellular respiration rate in the mitochondria can continue unhindered even under conditions of stress (25). tNIRS also causes NO to be released from other binding stores. NO is an important cellular signaling molecule, and is therefore involved in many physiological processes. Its binding action causes vasodilatatory effects through its potentiation of receptors found in vascular smooth muscle cells and most other cells in the body, which plays a central role in the cyclic guanine monophosphate (cGMP) signaling pathway and intracellular calcium concentrations (26), It is also a potent neurotransmitter in the central nervous system (CNS), which is capable of inducing synaptic plasticity due to long-term-potentiation (LTP) (27).

The reactive oxygen species (ROS)-pathway is another possible intracellular pathway modulated by tNIRS. Increased cellular respiration and increased oxygen consumption follow rises of intracellular ROS (28), which in turn, increases the overall redox potential of the cell. This plays an important regulatory role in DNA expression, protein synthesis and modification of enzyme and cell cycle activity (29).

We have observed that the tNIRS-driven cortical excitability decrease facilitates the implicit learning process only in female subjects, at least demonstrated by the SRTT task. Previous studies suggest that an excitability enhancement and not a decrease coincides with a facilitation of the learning process by inducing the strengthening of synapses and inducing long-term potentiation via the modulation of NMDA-receptor efficacy (e.g. (30). Indeed, prior studies using excitatory electrical stimulations (anodal transcranial direct current (tDCS) and random noise stimulation (tRNS)) reported enhanced learning during stimulation (31, 32). However, female subjects had a higher error rate while executing the task, even in the sham condition. Although no gender specific differences have been found for the molecular and cellular mechanisms of cortical stimulation, e.g. how tissue responds to light stimulation, our results suggest that the induced neuroplastic changes in the cortex might be gender specific. Previous studies indicate that differences do exist between the genders where transcranial electrical stimulation is applied (33, 34). In women excitatory increasing anodal stimulation heightened visual cortical excitability significantly when compared to age-matched male subjects (34). In the M1 female subjects showed prolonged after-effects of inhibitory cathodal stimulation, while male subjects showed stronger anodally-induced after effects (33). Further studies are necessary to clarify the role of hormones in this context. The other possibility is that the gender specific skull thickness (35) modified the neuroplastic effect of the tNILS. It is well documented that the penetration deepness of the infrared light depends on the thickness of the scalp and skull (e.g. (36, 37)). Due to the thicker skull (38) that results in higher level of light scattering and longer pathway in female subjects might have resulted in stimulation of

cortical areas, those functions were more relevant in motor learning. However, we did not see a gender specific effect of tNILS on MEP amplitudes in the single and paired-pulse experiments. Nevertheless, in MEP measurements and in implicit motor learning different anatomical pathways and physiological processes are involved that might reflect the involvement of diverse neuronal populations. tNILS may predominantly excite area 4p (old M1 at the crown), whereas TMS may predominately activate 4a (new M1 in the wall of the precentral gyrus) (39-41). Assuming that TMS primarily targets cortical columns of 4a (42) soma-depolarizing, anteriorly directed currents are best for low thresholds, whilst soma hyperpolarizing/dendrite-depolarizing currents are optimal for plasticity induction. Also in the original study on implicit motor learning under tDCS (31) cortical excitability defined by MEP decrease after cathodal and MEP increase after anodal stimulation differed from the effect of tDCS on motor learning: here both anodal and to lesser extent cathodal stimulation led to a reduction in reaction times.

One of the limitations of this study is that a high percentage of participants reported cutaneous perceptions, including burning and pain during stimulation and therefore, were able to differentiate between the active and sham stimulation conditions, which might influence the present results. Indeed, in an earlier study, suppression of MEPs was previously observed, after painful infusion of hypertonic saline into the hand muscle (43); nevertheless, here acute pain was induced in the muscle from which the MEPs were recorded. On the other hand positive and negative emotions (like pain) (44) and increased attention toward the experimental procedure (45) have been suggested to increase MEP size. Although, in our control condition we did not experience any MEP size change, further work should develop a better placebo condition. Besides this we have to minimize any accompanying cutaneous sensations.

In summary, recent human and animal studies have shown that near-infrared light applied over the cortex may have beneficial effects on stroke rehabilitation and may minimize

cognitive deficits sustained during traumatic brain injury (4, 5, 46). Here, we claim that tNIRS offers the potential to induce neuroplastic changes in the intact human cortex with a high spatial resolution and with good focality. Since tNIRS is believed to modify mitochondrial respiration, it might offer a possibility to aid in the management of a wide variety of disease pathologies originating from mitochondrial dysfunction.

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Conflict of interest: The authors have no conflicts of interest, financial or otherwise.

Table 1. Results of the ANOVA for the SICI/ICF measurements.

Factor	**df**	**F**	**p**
Condition	1,12	7,9891	*0,015275*
Time	2,24	0,4251	0,658552
ISI	4,48	63,8137	*0,000000*
Condition x Time	2,24	0,9947	0,384577
Condition x ISI	4,48	1,2716	0,294202
Time x ISI	8,96	0,7156	0,677245
Condition x Time x ISI	8,96	1,5613	0,146715

Table 2. Results of the ANOVA for the LICI measurements.

Factor	**df**	**F**	**p**
Condition	1,14	0,04587	0,833505
Time	2,28	0,21111	0,810965
ISI	3,42	5,23893	*0,003664*
Condition x Time	2,28	0,04134	0,959559
Condition x ISI	3,42	1,03149	0,388435
Time x ISI	6,84	0,37226	0,894678
Condition x Time x ISI	6,84	0,59882	0,730486

Table 3. Results of the ANOVA for the recruitment curve (I/O) measurements.

Factor	**df**	**F**	**p**
Condition	1,14	0,0099	0,922050
Time	4,56	1,9594	0,113315
Stimulator output	2,28	71,0358	*0,000000*
Condition x Time	4,56	0,4700	0,757457
Condition x Stimulator output	2,28	0,2679	0,766919
Time x Stimulator output	8,112	2,4703	*0,016685*
Condition x Time x Stimulator output	8,112	1,9019	0,066497

Table 4: Perceptual and side effects of the stimulation. Mean intensity is displayed with SEM.

	During Stimulation							**After Stimulation**						
	Tingling				Itching sensation			Tingling				Itching sensation		
	N	%	MI		N	%	MI	N	%	MI		N	%	MI
active	7	47	2,6±0,7		2	13	3	2	13	1		2	13	2,5±0,5
sham	1	8	3		0			1	8	2		2	16	2
	Burning sensation				Pain			Burning sensation				Pain		
	N	%	MI		N	%	MI	N	%	MI		N	%	MI
active	15	100	3,5±0,5		9	60	3,3±0,6	4	26	2,0±1		4	26	3±0,5
sham	1	8	2		0			1	8	2		0		
	Headache				Fatigue			Headache				Fatigue		
	N	%	MI		N	%	MI	N	%	MI		N	%	MI
active	1	7	4		4	27	1,3±0,5	3	20	1,5±0,5		6	40	2±0,6
sham	1	8	1		5	38	2±0,5					4	32	1,9±0,5
	Unpleasantness				Nervousness									
	N	%			N	%	MI							
active	8	53			3	20	2,6±0,4							
sham	0				1	8	1							
	Change in visual perception							Change in visual perception						
	N	%	MI					N	%	MI				
active	1	7	2					1	7	2				
sham	2	16	2					1	8	2				

Figure legends

Figure 1. *tNIRS head montage.* The laser acupuncture needles are fixed to scalp with the crown and the bendable wire holding mechanism. The waves are carried via optical fibers to the stainless steel percutaneous needles.

Figure 2A. Effect of 10 min tNIRS on motor evoked potentials. Time course of motor cortex excitability changes for 30 minutes post-stimulation, shown after 10 min tNIRS over M1. The figure shows mean amplitudes and their SEMs. Asterisks indicate significant differences between MEP amplitudes after 5, 10-60 min post-stimulation compared to baseline.

Figure 2B,C. Individual MEP data after active and sham stimulation.

Figure 3. Effect of 10 min tNIRS on SICI/ICF. The figure shows mean amplitudes and their SEMs. Asterisks indicate significant differences between MEP amplitudes after 0 min post-stimulation compared to baseline.

Figure 4. tNIRS of the primary motor cortex improves implicit motor learning in female subjects. Reaction times decrease faster in the active condition when compared to the sham stimulation condition. The asterisk indicates a significant difference regarding reaction time differences between active and sham stimulation.

Figure 5. tNIRS of the primary motor cortex increased the number of errors made by female subjects during execution of the task.

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