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Induction of long-term potentiation-like plasticity in the primary motor cortex with repeated anodal transcranial direct current stimulation – Better effects with intensified protocols?

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ABSTRACT

Background: A single session of anodal tDCS induces LTP-like plasticity which lasts for about 1 h, while repetition of stimulation within a time interval of 30 min results in late-phase effects lasting for at least 24 h with standard stimulation protocols.
Objective: In this pilot study, we explored if the after-effects of a recently developed intensified single session stimulation protocol are relevantly prolonged in the motor cortex by repetition of this intervention.
Methods: 16 healthy right-handed subjects participated in this study. The effects of an intensified (3 mA-20min) and a standard anodal tDCS protocol (1 mA-15min) with short (20 min) and long (3 h) repetition intervals were compared with the effects of respective single session tDCS protocols (3 mA-20min, 1 mA-15min, and Sham). Cortical excitability alterations were monitored by single-pulse TMS-elicited MEPs.
Results: Compared to sham, both single session tDCS protocols (1 mA-15min, and 3 mA-20min) resulted in cortical excitability enhancements lasting for about 30 min after stimulation. The short repetition interval (20 min) resulted in a prolongation of after-effects for the standard protocol, which lasted for more than 24 h after stimulation. For the intensified protocol, the prolongation of after-effects was limited to 120 min after stimulation. The long repetition interval (3 h) resulted in no excitability-enhancing after-effects for the intensified, and only minor excitability enhancement within the first 30 min after the intervention for the standard protocol.
Conclusion: These results suggest a non-linearity of late-phase LTP-like plasticity induction, which was dependent not only on the interval of intervention repetition, but also on other protocol characteristics, including intensity, and duration of tDCS. Further studies in larger samples are needed to confirm these results.

1. Introduction

Neuroplasticity is the foundation of a multitude of cognitive and behavioural processes in health and disease, including memory acquisition, learning, and the recovery from neurological injury. It refers to the experience-dependent enduring modification of the structure and function of neuronal connectivity. Functional, or synaptic plasticity has been extensively studied in animal, slice, and cellular models, and involves strengthening or weakening of synaptic connections, referred to as long term potentiation (LTP), or depression (LTD) [1]. Whereas a relatively transient form of LTP, and LTD, which lasts for a few hours after intervention, was explored most extensively so far, and is labelled early LTP/LTD (E-LTP/LTD), longer-lasting LTP, and LTD (late LTP/LTD, L-LTP/LTD) have been less frequently studied [2]. The latter is thought to be especially relevant

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for cognitive processes such as learning and memory formation, which require enduring alterations of cerebral functions for stable storage of information. Both forms of synaptic plasticity differ with respect to underlying mechanisms. E-LTP of glutamatergic synapses, which are the main excitatory synapses in the brain, is induced by relatively short single sessions of high frequency, or patterned stimulation [1]. This intervention results in calcium influx via *N*-methyl-D-aspartate (NMDA) receptors into the postsynaptic cell, which leads to the formation of the calcium/calmodulin kinase II (CaMKII) complex that activates protein kinase C (PKC), and ultimately results in the phosphorylation of AMPA receptors, and insertion of NMDA, and AMPA receptors into the subsynaptic membrane. The enhanced availability, and activity of AMPA receptors increases postsynaptic depolarization as response to a presynaptic stimulus, and thus strengthens synaptic connections. Respective effects last usually not longer than 3 h after respective plasticity induction [1]. Late-phase LTP is usually induced by repeated high frequency stimulation, spaced by intervals of some minutes [3]. These stimulation protocols activate cAMP-dependent protein kinase A (PKA), cAMP-response element binding (CREB) protein, messenger ribonucleic acid (mRNA) and protein synthesis, which ultimately leads to the production and insertion of new NMDA and AMPA receptors into synaptic membranes, thereby enhancing synaptic efficacy for much longer durations [3–6].

Plasticity-inducing interventions are available also for application in humans, which include transcranial electrical, and magnetic stimulation techniques [7,8]. Standard protocols induce cortical excitability alterations lasting for about up to 1 h and are thus in the range of E-LTP [9]. As far as this can be studied in intact humans, mechanisms of action are similar to those obtained in animal models, including the involvement of calcium-dependent processes, and glutamatergic NMDA receptors [10]. Respective stimulation protocols have been shown to affect cognitive and behavioural processes in health and disease. Especially for clinical application, it would be advantageous to develop interventions, which induce more stable effects, including L-LTP. Similar to animal models, here, spaced stimulation holds promise [11].

Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation (NIBS) tool which induces plasticity via the application of weak direct currents over the scalp, which results in current flow in the brain. Primary effect is a polarization of neuronal membranes, which depends on electrical field orientation, and results in enhanced or reduced neuronal excitability, and activity [12,13]. Stimulation for some minutes results in respective neuroplastic after-effects [12] which depend on the influx of calcium ions through glutamatergic NMDA receptors [14–16], and involves GABAergic de-activation as a gating mechanism for tDCS-induced glutamatergic transmission [17,18]. After-effects of a single session of tDCS with conventional parameters last for up to a few hours, and are well within the range of E-LTP [19]. Efforts have been made to extend stimulation effects to the L-LTP range by increasing stimulation intensity, and duration. However, extending the duration of stimulation with a given intensity resulted in conversion of excitability enhancement into diminution [20]. This indicates that single intervention tDCS is not well suited to induce L-LTP. Based on respective animal models, which showed that L-LTP can be induced by repeated stimulation with intervals of 30 min or less, the efficacy of spaced tDCS to induce respective after-effects was probed recently. Here, application of a standard stimulation protocol (13 min anodal tDCS with 1 mA intensity) with intervals of 3 and 20 min, but not 3, and 24 h, resulted in L-LTP-like plasticity of the human motor cortex [20]. These results are in accordance with those obtained for other NIBS protocols, including paired associative stimulation, and repetitive transcranial magnetic stimulation [11,20,21]. Thus, spaced stimulation within specific time intervals is a promising approach to induce L-LTP-like plasticity in humans.

We have recently shown that extending intensity, and duration of a single session of anodal tDCS to 3 mA, and 20 min improves efficacy of the intervention, as compared to standard protocols. Specifically, in that study, we expanded and titrated the parameter space of tDCS, with current intensities of 1, 2, and 3 mA, and stimulation durations of 15, 20, and 30 min, compared with sham stimulation (1 mA, 15 s) [22]. We found that for the nine intensity-duration combinations tested, the 3 mA-20min condition was the protocol with the most prominent neuroplastic changes when compared to sham. Neuroplastic effects however were still in the E-LTP range [22]. In the present study we were interested to explore if such intensified protocols result also in improved L-LTP-like plasticity, if appropriate spaced protocols are applied. Considering the respective previous studies, we hypothesized that repeated intervention with short intervals induce L-LTP, and the size of respective effects will be larger for an intensified stimulation protocol. In contrast, repeated stimulation with long intervals, and a single stimulation session – independent from the specific protocol - should not result in L-LTP-like plasticity.

2. Methods

2.1. Participants

Sixteen right-handed non-smoking healthy subjects (11 females, mean age = 26.5 ± 2.5 years) participated in this pilot study. All volunteers were examined by a physician to determine their overall health state. This included a thorough review of the participants' health state using a checklist of exclusion criteria; participants with a history of neurological or psychiatric disorders or any metal implants in their head or brain, or currently taking central nervous system-acting medications were excluded. To minimize confounding variables and keep a stable baseline MEP across all sessions, conditions of the participant that could affect the experimental outcome were kept constant. Participants were advised to maintain a proper sleeping routine, and to avoid strenuous physical activity for at least 24 h before each experimental session. Consumption of coffee was not allowed for at least 2 h before the start of each experimental session. Session order was randomized, and participants were blinded to the stimulation conditions they received.

The ethical committee of the Leibniz Research Centre for Working Environment and Human Factors (IfADo) approved this study, which agrees with the provisions of the Declaration of Helsinki [23]. All participants gave written informed consent before participation and received financial compensation.

2.2. DC stimulation of the motor cortex

DC was supplied by a battery-powered stimulator (The NeuroCare Group, Germany) via a pair of carbonated rubber electrodes which were covered by sponges (size 7 × 5 cm) soaked in physiological saline solution. The anode was positioned over the left primary motor cortex (M1) representation area of the abductor digiti minimi muscle (ADM) determined by TMS [12]. The reference electrode was positioned over the right supra-orbital area. tDCS was applied with a standard (1 mA-15min) and an intensified (3 mA-20min) protocol. For repeated session conditions, both protocols were repeated after an inter-stimulation interval of 20 min or 3 h. Sham tDCS was conducted with 1 mA applied for 15 s, with the stimulation setup kept on the head for 15 min to simulate an active stimulation condition [24]. The standard protocol was based on previous studies which found excitability alterations

lasting for up to an hour after stimulation for related protocols [9,22,25]. For the intensified protocol (3 mA), our previous titration study with single session protocols showed better effects when compared to the standard protocol [22]. The repetition intervals of 20 min, and 3 h were also based on a previous study in the motor cortex which found late-phase LTP-like plasticity with a standard protocol (1 mA anodal tDCS) for the short interval (20 min), and not for the long (3 h) repetition interval [20]. In animal models, late-phase LTP was induced by two trains of high frequency theta burst stimulation spaced with an inter-stimulation interval of 30 min or less [2].

For both, sham and active stimulation conditions, current was ramped up and down for 10 s each. A topical analgesic cream (EMLA, 2.5% lidocaine and 2.5% prilocaine) was applied to the scalp before each tDCS session to blind participants for the stimulation condition, and to minimize discomfort associated with higher intensities of stimulation.

2.3. Measurement of motor evoked potentials

MEPs were recorded by surface gold-plated electromyography (EMG) electrodes attached to the right ADM in a belly-tendon montage and induced by a transcranial magnetic stimulator (Mag and More, Munich, Germany). TMS pulses were delivered at 0.25 Hz (with 10% jitter) through a standard figure of eight coil with a diameter of 70 mm, and a maximum magnetic field of 2 T. The coil was held above the motor cortex, touching the scalp tangentially with the handle pointing backwards at about 45° to the midline, and current flowing in the posterior-anterior direction. The optimal area in the motor cortex representing the ADM, where TMS of a given medium intensity produced the highest averaged MEPs, was identified and marked (motor hotspot). Then the TMS intensity that elicited MEPs of about 1 mV amplitude was identified. EMG signals recorded from the ADM were sampled at 5 kHz (CED, Cambridge, UK), amplified and band pass filtered at 2 Hz–2 kHz (Digimeter, Hertfordshire, UK) using the Signal software (version 6.0) and stored offline for further analyses.

2.4. Procedure of the experiment

Participants were seated in a comfortable adjustable reclining chair, with an inflatable pillow for keeping the neck relaxed and head stabilized during the experiment. Each experimental session started with the identification of the motor hotspot. The stimulation intensity (SI) of TMS was then adjusted until it produced peak-to-peak MEP amplitudes of approximately 1 mV (SI_{1mV}) and was kept constant throughout the remaining session. To obtain baseline cortical excitability, 25 MEPs were recorded and averaged. Then tDCS was applied. After-effects of tDCS were measured by recording 25 MEPs with the same SI_{1mV} intensity immediately after tDCS with 5 min intervals until 30 min, and then every 30 min until 2 h. In addition to this, after-measures were taken the same evening (SE), next morning (NM), next noon (NN), and next evening (NE) (see Fig. 1 below).

We administered a modified questionnaire based on [26] to obtain information about side-effects of tDCS, and blinding efficacy.

2.5. Data analysis and statistics

Participants were pre-tested before inclusion in the study to explore if it was possible to obtain the MEP size of 1 mV required for baseline excitability measures. A 1-h test session was conducted for each participant to expose them to TMS and tDCS, to check for adequate size of baseline MEPs, and reduce first exposure-related arousal, which would have some probability to affect results. 9 participants were rejected due to insufficient baseline MEP amplitude size obtainable by TMS.

Peak-to-peak MEP amplitudes at each time point pre- and post-stimulation were recorded, averaged, and normalized to baseline for each individual. MEPs with muscle artefacts were removed before averaging.

To exclude differences between baseline MEPs and SI1mV for all 7 sessions of the experiment, a one-way repeated measures ANOVA was conducted with baseline MEPs and SI1mV as dependent factors and session as a within-subject factor.

Time frames for all conditions were pooled into four epochs: baseline (BL), and post-stimulation early (0–30 min), late (60–120 min), and very late (SE-NE) epochs, to compensate for variability between single time bins, and for comparability between studies. To investigate if changes of cortical excitability post-tDCS differed between conditions, a two-way repeated measures ANOVA was conducted for these pooled data, with Condition (7 levels) and Epoch (4 levels) as repeated measures factors, and normalized MEPs as dependent variables. In case of significant effects of the ANOVAs, exploratory post-hoc Student's t-tests (paired samples, two-tailed, not corrected for multiple comparisons) were conducted to examine differences between baseline and post-tDCS MEPs, between active and sham sessions, and between respective active single, and repeated stimulation sessions.

For statistics on non-epoched data, please refer to supplementary materials (S3, Table 2).

To explore if common side-effects of tDCS reported by participants differed between sessions, a one-way repeated measures ANOVA was conducted with Condition (7 levels) as repeated measures factor and side-effect as dependent variable. In case of a significant effect, a one-tailed post-hoc *t*-test was conducted to examine differences between the conditions with respect to that side-effect. For significant side-effects, a Pearson correlation coefficient was furthermore calculated to explore the association between these side-effects and epoched MEP amplitudes.

Mauchly's test of sphericity was computed for all ANOVAs, and where necessary the Greenhouse-Geisser correction was applied. All analyses were carried out using SPSS version 24 (IBM SPSS Statistics, New York, USA).

3. Results

Apart from some tingling, burning sensations, pain, and headache, which are often reported with tDCS studies [22,24,26], the stimulation was well tolerated by all participants (Tables 4 and 5), and blinding was successful (Table 3).

3.1. Baseline measures and SI_{1mV}

The results of the respective ANOVAs show no significant differences of baseline MEPs between the 7 sessions (Table 1, supplemental material, S2).

3.2. After-effects of the interventions measured within the epochs

The results of the respective ANOVA show significant main effects of Condition, and Epoch, and a significant interaction between Condition and Epoch (see Table 2).

Pairwise comparisons of the single session protocols with their respective baselines and sham show an enhancement of cortical excitability only in the early epoch (Fig. 2A). Post-hoc comparisons of the short repetition interval conditions show that the standard protocol significantly enhanced excitability in the early, late, and very late epochs when compared to baseline. The intensified protocol shows excitability enhancement in the early and late epochs

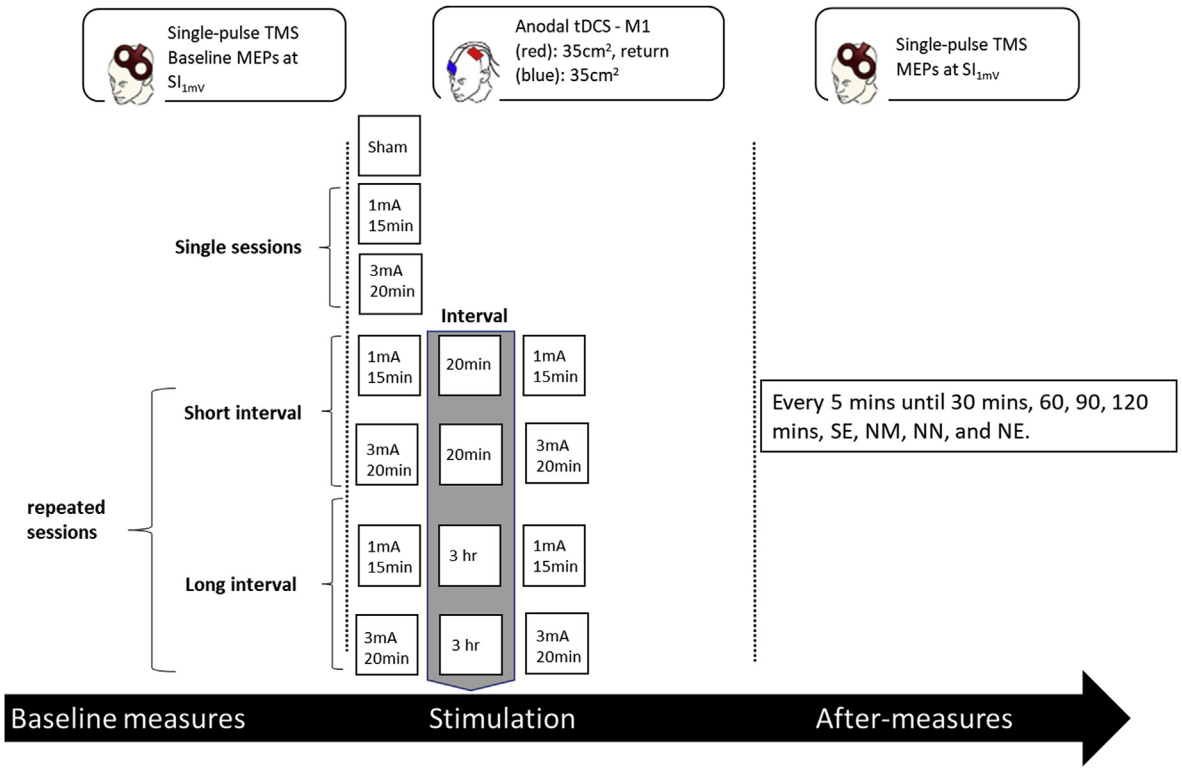

Fig. 1. Diagrammatic representation of the experimental procedure.
First, baseline measures of cortical excitability were obtained for each participant in all sessions (25 MEPs recorded with SI_{1mV}). tDCS was then applied depending on the stimulation condition – single or repeated, and immediately after that cortical excitability measurements were obtained every 5 min until 30 min, and then every 30 min until 120 min. After-measurements were also obtained on the same day evening (SE), next morning (NM), next noon (NN), and next evening (NE) after tDCS.

when compared to baseline (Fig. 2B). For the comparisons with sham, post-hoc tests show that both, standard and intensified protocols, significantly increased cortical excitability in the early and late epochs (Fig. 2B). Pairwise comparison of long interval repetition conditions with their respective baselines show no significant differences of cortical excitability, however when compared with sham, the standard protocol shows a significant enhancement of cortical excitability in the early epoch (Fig. 2C).

For an overview of the non-epoched data, please refer to Fig. 3(A–C), and the supplemental material S3.

3.3. Blinding of participants to tDCS and side effect ratings

To investigate the effectiveness of blinding, Chi-Square tests were computed to evaluate differences between wrongly and correctly guessed tDCS-intensities.

The results of the overall Chi-Square test indicate no significant differences between responses of participants with respect to the guessed tDCS intensities received ($\chi^2_{1,\ 112} = 4.625$, $p = 0.099$).

Numerically, side-effect ratings in this study were low across all 11 side-effects reported (Table 3). For tingling, the highest mean value recorded was 1.68 (on a scale of 0–5), whereas for burning sensation it was 1.43, for pain 0.81, and for headache it was 1.50.

Post-hoc comparisons of the side-effect ratings were conducted for the 4 significant side-effects for all 6 real stimulation conditions vs sham tDCS. For tingling, the 3 mA-20min.20min.3 mA-20min condition showed significantly higher ratings ($p = 0.041$). For burning sensation, 3 mA-20min ($p = 0.012$), and 3 mA-20min.20min.3 mA-20min ($p = 0.007$) showed significantly higher ratings when compared to sham. For pain, 1 mA-15min.3hr.1 mA-15min ($p = 0.004$), 3 mA-20min ($p = 0.026$), 3 mA-20min.20min.3 mA-20min ($p = 0.005$), and 3 mA-20min.3hrs.3 mA-20min ($p < 0.001$) significantly increased pain

Table 1
Baseline MEPs and TMS intensity for 1 mV MEP (SI_{1mV}) of all 7 sessions of the experiment. There was no significant difference between the baseline MEPs and TMS intensities of the 7 sessions of the experiment. Values in the table represent mean ± SD of baseline excitability (Baseline MEP) and TMS intensity (SI_{1mV}). MSO – maximum stimulator output.

Repetition interval	Condition	Baseline MEP (mV)	SI_{1mV} (% MSO)
	Sham	1.02 ± 0.08	60.25 ± 11.11
Single session (No repetition)	Standard protocol	1.01 ± 0.10	60.25 ± 11.05
	Intensified protocol	0.99 ± 0.06	60.19 ± 10.42
Short Interval	Standard protocol	1.06 ± 0.12	61.06 ± 13.04
Repetition (20 min)	Intensified protocol	1.02 ± 0.09	60.56 ± 12.12
Long Interval	Standard protocol	1.03 ± 0.11	60.69 ± 10.85
Repetition (3 h)	Intensified protocol	1.05 ± 0.11	59.31 ± 11.76

Table 2
Table showing the ANOVAs conducted for epoched data. Results of the repeated measures ANOVA conducted for condition, epochs, and the respective interactions to identify intervention-dependent motor cortex excitability alterations.

	Measurement	df	F value	η^2p	P value
Parameters	Condition	3.284 (90)	4.651	0.237	0.005*
	Epoch	3 (45)	10.119	0.403	<0.001*
	Condition x Epoch	18 (270)	2.615	0.148	<0.001*

*$p < 0.05$. η^2_p = partial eta squared.

ratings when compared to sham. Finally, for headache, the 1 mA-15min.20.1 mA-15min ($p = 0.048$), and 3 mA-20min.20min.3 mA-20min conditions resulted in higher ratings compared to sham ($p = 0.006$).

A Pearson correlation was computed for these four side-effects to explore associations between side-effects and MEPs recorded post-tDCS. Since side-effects were obtained immediately after the late epoch (first 2 h after tDCS), and at the very late epoch (24 h after tDCS), we re-epoched MEPs in the early and late epochs into a single epoch, to correspond with the timing of the measure of side-effects. We found significant correlations only for pain and tingling. For pain, a significant negative correlation was found for averaged MEPs of the sham ($r = -0.479$, $p = 0.030$), 1 mA-15min ($r = -0.437$, $p = 0.045$), and 3 mA-20min conditions ($r = -0.585$, $p = 0.009$). For tingling, a significant negative correlation was found only for the 3 mA-20min condition ($r = -0.559$, $p = 0.012$). No correlations were however found between all four critical side-effects and MEPs recorded in the very late epoch.

4. Discussion

We examined the feasibility to induce late-phase LTP-like plasticity in the motor cortex of healthy adults via repeated stimulation with anodal tDCS by use of standard versus intensified intervention protocols. Single session anodal tDCS with both, a standard (1 mA-15min) and an intensified (3 mA-20min) protocol resulted in significant enhancements of cortical excitability that lasted for 30 min post-tDCS. For repeated sessions, the short repetition interval of 20 min significantly enhanced cortical excitability until the next day after stimulation for the standard tDCS protocol, while the intensified protocol enhanced cortical excitability for not more than 120 min. For the long repetition interval of 3 h, only the standard protocol increased MEP amplitudes slightly. Thus, we were able to induce late-phase LTP-like plasticity by a standard, but not an intensified tDCS protocol, and this effect depended critically on the duration of the interval between interventions, but involved also a contribution of stimulation intensity, and/or duration.

Both single session tDCS conditions resulted in motor cortex excitability enhancements, which lasted for about 30 min, similar to a recently conducted titration study [22]. In further accordance, the intensified protocol resulted in slightly enhanced MEP amplitudes, as compared to the standard condition. This time range of respective excitability alterations is within that of previous tDCS studies [9,22,25], and most likely represents E-LTP-like plasticity. Minor differences between studies might be caused by inter-individual heterogeneities of stimulation effects [27]. Respective after-effect durations are furthermore a common pattern of results obtained by other NIBS protocols suited to induce LTP-like plasticity, such as paired associative (PAS), repetitive transcranial magnetic stimulation [28–30], and animal models of plasticity [1,2]. In contrast to the results of the single tDCS intervention protocols, repetition of tDCS with a short interval of 20 min significantly enhanced the after-effect duration for more than 24 h with the standard tDCS protocol which is in accordance with induction of L-LTP-like plasticity, based on the time-scale of the after-effects. This furthermore replicates the results of a previous tDCS study conducted with a similar protocol [20]. In that study, short repetition intervals of 3 and 20 min, both within the critical time frame for the induction of late-phase LTP, increased the duration of after-effects until next evening after stimulation. Importantly, in the present pilot study, this effect was observed for the 1 mA stimulation protocol, which was also applied in the previous study, but not for the intensified 3 mA stimulation protocol. For the latter, respective after-effects were only present for about 120 min, and thus in the range of E-LTP. Thus, a kind of non-linearity was observed for the induction of late phase plasticity, which probably depends on characteristics of the stimulation protocol, with respect to stimulation intensity, and/or duration. The inducibility of L-LTP-like plasticity was also suggested for other NIBS protocols with similar intervals. Two trains of intermittent theta-burst stimulation (iTBS) delivered with an inter-stimulation interval of 15 min enhanced MEP amplitudes to a larger degree, and longer duration than single iTBS [31]. In another study, two PAS_{LTP} protocols delivered within a time window of 30 min, but not longer intervals, enhanced MEP amplitudes [21]. These results are furthermore compatible with those of animal experimentation [2,32]. The longer repetition interval of 3 h did not lead to L-LTP, instead this protocol induced only minor or no excitability enhancements, especially with respect to the intensified stimulation protocol. This confirms the results obtained in the previous study of Monte-Silva and colleagues who found no significant increases of cortical excitability for repetition intervals of 3, and 24 h [20]. Similar findings were described for PAS, where inter-stimulation intervals of 60, 120, and 180 min did not significantly enhance MEP amplitudes [21]. These results are furthermore in principal agreement with evidence from animal models, that suggests that shorter inter-stimulation intervals are better suited for L-LTP induction by spaced stimulation [2,33]. Woo and Nguyen induced L-LTP in hippocampal slices with a spaced stimulation protocol of 5 min inter-stimulation interval [33], and in the intact free moving animal, a maximum stimulation interval of 30 min is required for L-LTP induction [32].

4.1. Proposed mechanisms

Given the different and nonlinear neuroplasticity effects of the tDCS protocols under study, which probably depended on the intensity of the stimulation protocols, as well as the inter-stimulation interval, mechanistic explanations are required. E– and L-LTP, which are suggested to be the foundation of these effects, differ by respective biochemical pathways. In animal experiments, for E-LTP, a single high-frequency stimulation causes calcium influx through glutamatergic NMDA receptors leading to the activation of calcium/calmodulin kinase II, which activates PKC, and ultimately leads to the phosphorylation of AMPA receptors. This process results in a larger number of receptors available for depolarization of downstream postsynaptic cells [2,34]. Whereas phosphorylation of AMPAR is observed in both, E– and L-LTP, E-LTP uses the rapidly depleted PKC and CaMKII pathways to transport phosphorylated AMPA receptors to the synaptic membrane [6], therefore making it susceptible to depotentiation, and limiting its duration to a few hours [35]. Plasticity induced by single session tDCS with conventional protocols, like the one in this pilot study, is suggested to represent E-LTP due to the duration of after-effects [9], and the involvement of NMDA [15] and AMPA receptors [36,37]. In contrast, L-LTP is a product of transcription-based protein synthesis, which leads to more AMPA receptors being inserted into the postsynaptic cell membranes via persistent PKA and CREB pathways [2,6], and involves brain-derived neurotrophic factor synthesis (BDNF)

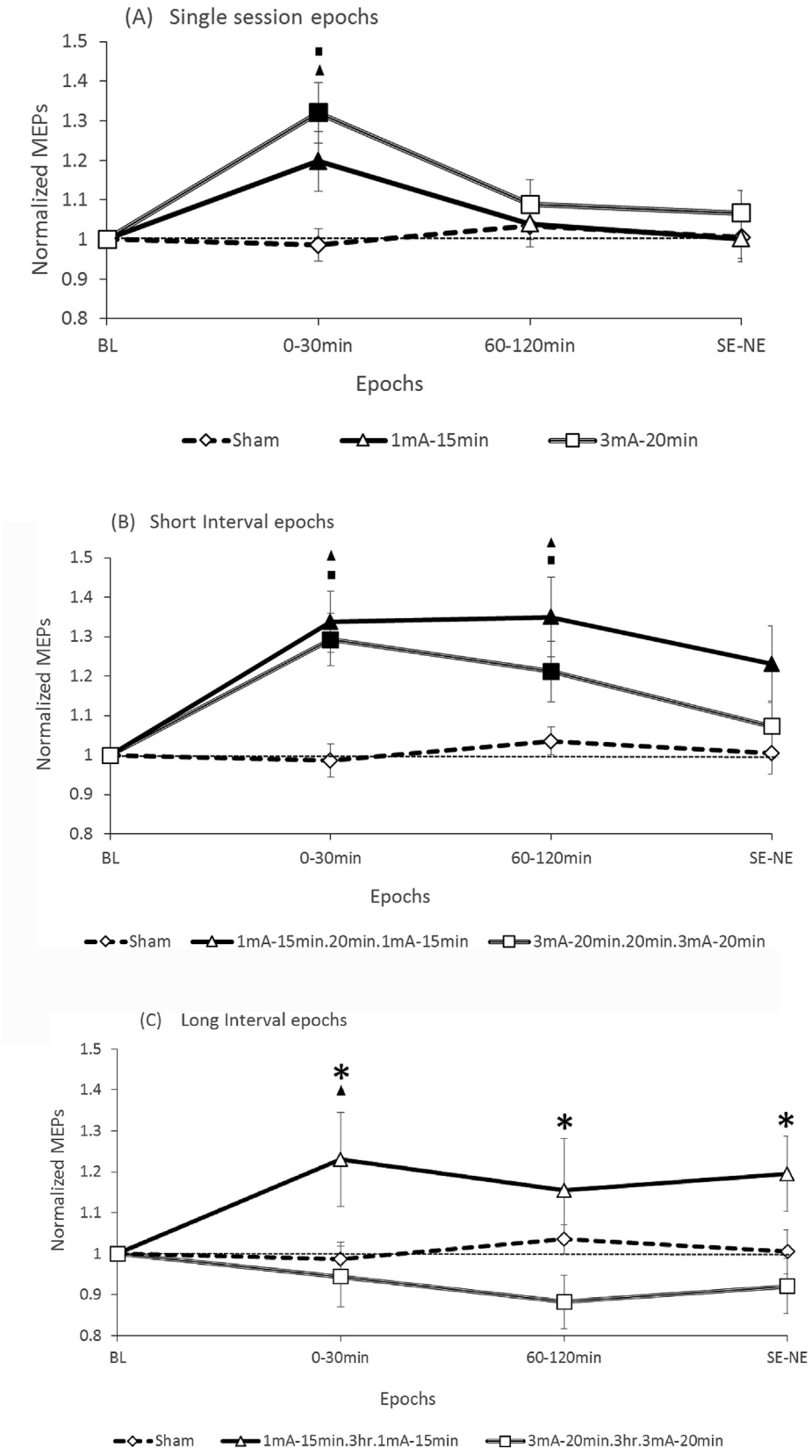

Fig. 2. Cortical excitability alterations induced by single session, and spaced stimulation with anodal tDCS, as obtained by TMS-generated MEP for epoched time bins.
For both single session stimulation conditions, a significant enhancement of cortical excitability, as compared to their respective baselines and to sham, was observed only in the early epoch (A). When compared to baseline, the short repetition interval (20 min) condition of the standard protocol shows significant excitability enhancements in the early, late, and very late epochs. The short repetition interval of the intensified protocol resulted in excitability enhancements only in the early and late epochs (B). For the long repetition interval (3 h) conditions, both, standard and intensified protocols show no significant enhancements of cortical excitability for all epochs when compared with respective baseline values (C), however a significant enhancement of excitability induced by the standard protocol for the early epoch was observed, when compared with the respective sham stimulation condition. Filled symbols represent a significant difference of MEP amplitudes compared to the respective baselines. Floating symbols represent significant differences between real and sham stimulation conditions, while * represents significant differences between the long repetition interval conditions of the standard and intensified protocols (C) (paired *t*-test, two-tailed, $p < 0.05$). Error bars represent the standard error of means. BL – Baseline, SE-NE – same day to next evening.

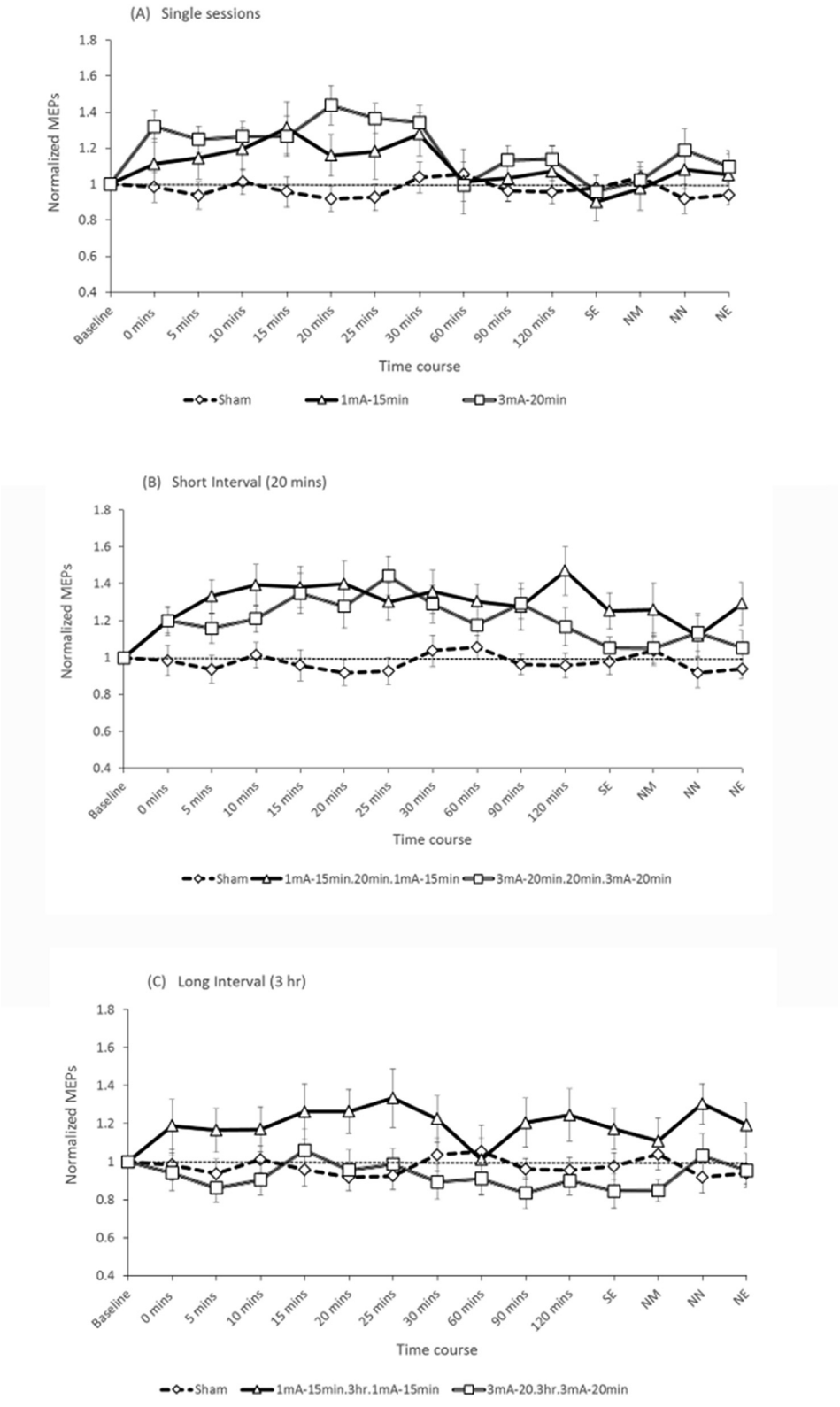

Fig. 3. Cortical excitability alterations induced by single session, and spaced stimulation with anodal tDCS, as obtained by TMS-generated MEP for all time bins.
For single session tDCS, after-effects lasted for up to 30 min, independent from the respective real stimulation protocol (A). For repeated sessions with a 20 min interval, relevantly longer excitability alterations were obtained especially for the standard stimulation protocol, which induced late-phase effects (B). The long repetition interval of 3 h resulted in a slight increase of cortical excitability for the standard protocol, but no excitability enhancement was observed for the intensified protocol (C). SE – same evening; NM – next morning; NN – next noon; NE – next evening. Error bars show standard error of means (SEM).

Table 3
Shown are the frequencies of actual and guessed tDCS intensities. The rows represent the actual intensity of stimulation, while the columns represent the intensity guessed by the participants. The difference of the number of ratings between conditions is due to the presence of only one sham condition protocol (1 mA, 15 min single intervention) as opposed to 3 conditions of real stimulation intensity (single, short, and long interval interventions).

		Guessed Intensity			
		0 mA	1 mA	3 mA	Total
Actual Intensity	Sham	5	8	3	16
	1 mA	13	19	16	48
	3 mA	9	18	21	48

[38,39], which is also relevant for tDCS effects [40,41]. Moreover, the synaptic tagging hypothesis is an attractive mechanism to explain how these newly transcribed mRNA and proteins are transported from the soma to the dendrites for plasticity induction. Accordingly, activated or 'tagged' glutamatergic dendritic synapses are able to 'capture' mRNA complexes if these synapses were previously activated by stimulation [42]. These synaptic tags however have a half-life of about 30 min [2]. This means that short intervals between repeated stimulation sessions are probably crucial for the efficient capture of mRNA complexes and synaptic proteins needed for L-LTP induction [2,42]. The timelines of the respective mechanisms fit well to the results obtained in the present study. The synaptic tagging hypothesis, however, needs to be experimentally validated in future studies in animal models for tDCS effects.

In the present pilot study, short interval repetition induced L-LTP-like plasticity only for the standard, but not for the intensified tDCS protocol. One possible explanation for this stimulation intensity-dependent non-linearity of effects might be that intensified tDCS has a stronger activating effect on NMDAR, and the resulting higher amount of calcium influx induces counterbalancing effects with a second stimulation [43,44]. Specifically, calcium overflow due to the higher stimulation intensity might activate potassium channels, thereby limiting the induction of L-LTP-like plasticity [43]. It should however be noted that these plausible explanations are insufficiently explored experimentally so far.

As expected, long repetition intervals of both, the standard and intensified protocols, resulted in no L-LTP-like plasticity, but a reduction or abolishment of after-effects. This fits well with the limited time-frame of the 'tagging' mechanism, as explained above. Respective stimulation protocols moreover reduced the ability of tDCS to induce E-LTP-like plasticity. This could probably be due to a homeostatic regulation of synaptic efficacy, which prioritizes the maintenance of a dynamic range of physiological activity [44], and might predominate outside the time frame for the induction of L-LTP, though other synaptic mechanisms cannot be completely ruled out, and should be explored systematically in future studies.

The Bienenstock-Cooper-Munro (BCM) rule postulates a sliding threshold of LTP or LTD induction, depending on the state of

Table 5
Results of the one-way ANOVAs conducted for the side-effects of tDCS. Shown are the results of the one-way repeated measures ANOVAs conducted for 11 commonly reported tDCS side-effects. The ANOVAs are based on the raw scores of side-effect ratings experienced during, and 24 h after tDCS. The side-effects were rated on a scale between 0 and 5 (0 – absence of side-effect, 5 – maximal side-effect). Tingling, burning sensations, pain, and headache ratings differed statistically significantly between conditions (critical $p < 0.10$). Because of the directed hypothesis of larger side effects in case of higher stimulation intensities, and to avoid an erroneous rejection of the hypothesis because of the relatively small sample size, we chose a liberal p-value of 0.10.

Time	Side-effects	df	F value	η^2_p	p
During tDCS	Visual	3.970 (59.545)	1.493	.091	.216
	Itching	6 (90)	.276	.018	.947
	Tingling	**3.238 (48.566)**	**2.388**	**.137**	**.076***
	Burning	**3.615 (54.266)**	**2.573**	**.146**	**.053***
	Pain	**2.847 (42.705)**	**3.000**	**.167**	**.043***
24 h after tDCS	Redness	3.035 (45.532)	1.912	.113	.140
	Headache	**6 (90)**	**2.007**	**.118**	**.073***
	Fatigue	6 (90)	1.781	.106	.112
	Concentration	6 (90)	1.824	.108	.103
	Nervousness	6 (90)	1.085	.067	.378
	Sleep	6 (90)	1.244	.077	.292

*$p < 0.10$. η^2_p = partial eta squared.

Table 4
Rating of side-effects during, and 24 h after stimulation. The table shows mean values ± sd of each side-effect as rated by participants on a Likert scale of 0–5, with 0 meaning the absence of a side-effect, and 5 indicating the highest intensity (mean ± SD).

Time	Side-effects	Sham	1 mA-15min	1 mA-15min. 20min. 1 mA-15min.	1 mA-15min. 3hr. 1 mA-15min.	3 mA-20min	3 mA-20min. 20min. 3 mA-20min.	3 mA-20min. 3hr. 3 mA-20min.
During tDCS	Visual	0.25 ±0.58	0.31 ±0.70	0.44 ±0.89	0.44 ±0.72	0.75 ±1.29	0.81 ±1.56	1.06 ±1.44
	Itching	1.00 ±1.03	0.81 ±0.91	0.94 ±0.85	1.13 ±1.02	1.06 ±0.68	1.00 ±0.82	1.00 ±0.89
	Tingling	0.94 ±0.93	0.81 ±0.75	0.88 ±0.81	1.00 ±0.82	1.44 ±1.31	1.68 ±1.49	1.44 ±1.15
	Burning	0.63 ±0.72	0.50 ±0.63	0.81 ±1.22	0.63 ±0.96	1.19 ±1.11	1.43 ±0.96	1.06 ±1.06
	Pain	0.13 ±0.34	0.19 ±0.40	0.38 ±0.81	0.50 ±0.73	0.81 ±1.28	0.81 ±0.98	0.81 ±0.83
	Redness	0.25 ±0.77	0.25 ±0.44	0.25 ±0.58	0.63 ±0.89	0.38 ±0.81	0.69 ±0.87	0.56 ±1.03
	Headache	0.50 ±0.89	0.81 ±0.98	1.06 ±1.24	0.94 ±1.06	0.69 ±1.14	1.50 ±1.37	1.00 ±0.97
24 h after tDCS	Fatigue	0.75 ±0.68	0.94 ±0.93	1.31 ±1.30	1.56 ±1.46	1.81 ±1.33	1.19 ±1.33	1.31 ±1.58
	Concentration	0.43 ±0.63	0.63 ±1.15	0.88 ±1.09	0.94 ±1.06	1.06 ±1.39	1.19 ±1.51	1.44 ±1.46
	Nervousness	0.13 ±0.34	0.13 ±0.34	0.19 ±0.45	0.25 ±0.45	0.25 ±0.45	0.38 ±0.62	0.19 ±0.40
	Sleep	0.25 ±0.68	0.13 ±0.34	0.69 ±1.40	0.75 ±0.93	0.38 ±0.62	0.56 ±1.03	0.75 ±1.44

previous neuronal activity [45]. Specifically, for already strengthened synaptic connections, the threshold for further LTP induction would be higher. Homeostatic plasticity has been also observed in other NIBS studies, where it was shown that the prior state of a cortical system affects the induction of LTP-like plasticity in line with the respective rules [46,47]. The counter-regulation of plasticity observed for the long interval in this study was moreover weaker for the 1 mA, as compared to the 3 mA stimulation condition. The higher stimulation intensity might have increased calcium influx, and neuronal activity to a larger degree, thereby raising the threshold of induction of LTP during the second stimulation more efficiently. Though a plausible explanation, to the best of our knowledge, no systematic mechanistic studies examining the different effects of periodic tDCS do exist. Further studies are therefore required to evaluate the relevance of calcium dynamics for the effects of periodic stimulation protocols.

4.2. General remarks

With the exception of tingling, burning sensations, pain, and headache, tDCS was tolerated by participants with respect to side-effects during, and 24 h after stimulation (Tables 4 and 5), which confirms previous reports of tolerability [48]. For the impact of side effects on the physiological outcome, it should be taken into account that for all stimulation conditions, side effects were lower than 2 on a Likert scale ranging between 0 and 5, and thus minor. Furthermore, we did see correlations between side effects, and MEP amplitudes for single session conditions, which were however negative, and thus do not indicate that larger side effects improved physiological effects. The negative relation might be caused by a distractive effect on attention, which has been shown to reduce efficacy of also other neuroplasticity-inducing interventions [49].

An assessment of the perception of respective tDCS conditions revealed that blinding was successful, as participants could not accurately guess the stimulation intensities they received (Table 3). Recently, there have been discussions on whether application of higher intensities could be useful, taking into account a higher probability for side effects [50]. The tolerability of 4 mA tDCS was recently assessed, albeit, with a novel experimental procedure, and shown to induce side-effects comparable to conventional protocols such as 2 mA [51,52]. Unlike the current study, these previous studies did not use a topical anaesthesia to reduce associated discomfort. For higher intensities of tDCS, the cost of more intense side-effects, if present, must be weighed against the benefits of the stimulation. In clinical applications, where the aim often is to alleviate symptoms, relatively higher side-effect ratings may be acceptable, if efficacy is improved by stronger stimulation. On the other hand, for cognitive, and behavioural studies in healthy participants, the use of higher intensities might not be necessary if side-effect ratings are high for these intensities, and efficacy of intensified effects is not better.

4.3. Limitations and future directions

Some limitations of this study should be taken into account. First, this was an exploratory study. Data were acquired from a relatively small sample of 16 participants over a couple of sessions involving different interventions. Thus, results are preliminary, and should be replicated in follow-up studies with larger samples. To reduce variability of MEP measures of the data set, we conducted the statistical analysis over epoched data for agglomerated time-points. Inferential statistics of non-epoched data are available in the supplemental material (S3). Furthermore, this study used young healthy participants, making an extrapolation of the results to older or clinical participants challenging, because the state of brain anatomy, and physiology might differ between respective populations, and this might impact on the specific neuromodulatory effects of tDCS.

Secondly, we did focus only on 20 min and 3 h repetition intervals based on previous studies. Further studies are needed to investigate whether very long repetition intervals such as 24 h or more would lead to similar effects, as suggested by the results of a previous study [20]. Exploring the transferability of these results from the motor to other cortical areas will be relevant, especially for clinical applications, but cannot be taken for granted. Spaced stimulation protocols have so far most extensively been studied in animal models. Pilot studies in humans, such as the present one, are rare, but show promising results with respect to prolongation of after-effects. Fine-tuning of respective stimulation protocols, such as increasing the number of repetitions [6], might be a promising approach to further enhance efficacy. Larger samples or multi-centre studies will be helpful to confirm the findings obtained in this exploratory study.

For cognitive, and behavioural applications, it remains furthermore to be determined how repeated stimulation interacts with task performance, and whether this combination will follow the same rules for the induction of late-phase plasticity in other cortical regions. A one-to-one transferability of these effects from the motor cortex to other cortical regions cannot be taken for granted. It is therefore important for a systematic evaluation of respective stimulation parameters to optimize effects for each cortical region. Finally, the mechanisms of late-phase plasticity are not fully explored so far, especially in humans, but also in animal models. Given the potential relevance of respective effects, gaining more detailed knowledge about mechanistic details would be highly important not only for basic sciences, but also clinical application.

5. Conclusions

We explored the induction of late-phase LTP-like plasticity with repeated standard and intensified anodal tDCS protocols in the motor cortex model of healthy humans. With single session stimulation, both, standard and intensified protocols induced E-LTP-like plasticity, based on the duration of after-effects. Repeated stimulation with a short interval (20 min) resulted in L-LTP-like plasticity for the standard protocol, while the intensified protocol only resulted in E-LTP. A longer repetition interval (3 h) did not induce L-LTP-like plasticity for both, standard and intensified protocols. The results from this pilot study suggest that L-LTP can be induced in the human motor cortex with repeated stimulation within relatively short, but not long intervals. They suggest moreover that the intensity of respective stimulation protocols might play an important role in the induction of L-LTP, in that moderately strong protocols might be superior. These results might help to define advanced protocols for inducing late-phase LTP-like plasticity in humans with repeated stimulation. Further studies with larger sample sizes are needed in the future to confirm the results from this exploratory study.

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Declaration of competing interest

Michael A. Nitsche is a member of the advisory boards of Neuroelectrics, and NeuroDevice. None of the remaining authors have potential conflicts of interest to be disclosed.

CRediT authorship contribution statement

Desmond Agboada: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft, Visualization. **Mohsen Mosayebi-Samani:** Data curation, Formal analysis, Writing - review & editing. **Min-Fang Kuo:** Conceptualization, Project administration, Supervision, Formal analysis, Methodology, Validation, Writing - review & editing. **Michael A. Nitsche:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2020.04.009.

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