PLOS | ONE

RESEARCH ARTICLE

Augmenting LTP-Like Plasticity in Human Motor Cortex by Spaced Paired Associative Stimulation

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Citation: Müller-Dahlhaus F, Lücke C, Lu M-K, Arai N, Fuhl A, Herrmann E, et al. (2015) Augmenting LTP-Like Plasticity in Human Motor Cortex by Spaced Paired Associative Stimulation. PLoS ONE 10(6): e0131020. doi:10.1371/journal.pone.0131020

Academic Editor: Guglielmo Foffani, Hospital Nacional de Parapléjicos, SPAIN

Received: February 6, 2015

Accepted: May 26, 2015

Published: June 25, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: The authors received no specific funding for this work.

Competing Interests: The authors declare that no competing interests exist.

Abstract

Paired associative stimulation (PAS_{LTP}) of the human primary motor cortex (M1) can induce LTP-*like* plasticity by increasing corticospinal excitability beyond the stimulation period. Previous studies showed that two consecutive PAS_{LTP} protocols interact by homeostatic metaplasticity, but animal experiments provided evidence that LTP can be augmented by repeated stimulation protocols spaced by ~30min. Here we tested in twelve healthy selected PAS_{LTP} responders the possibility that LTP-*like* plasticity can be augmented in the human M1 by systematically varying the interval between two consecutive PAS_{LTP} protocols. The first PAS_{LTP} protocol (PAS1) induced strong LTP-*like* plasticity lasting for 30-60min. The effect of a second identical PAS_{LTP} protocol (PAS_2) critically depended on the time between PAS_1 and PAS_2. At 10min, PAS_2 prolonged the PAS_1-induced LTP-*like* plasticity. At 30min, PAS_2 augmented the LTP-*like* plasticity induced by PAS_1, by increasing both magnitude and duration. At 60min and 180min, PAS_2 had no effect on corticospinal excitability. The cumulative LTP-*like* plasticity after PAS_1 and PAS_2 at 30min exceeded significantly the effect of PAS_1 alone, and the cumulative PAS_1 and PAS_2 effects at 60min and 180min. In summary, consecutive PAS_{LTP} protocols interact in human M1 in a time-dependent manner. If spaced by 30min, two consecutive PAS_{LTP} sessions can augment LTP-*like* plasticity in human M1. Findings may inspire further research on optimized therapeutic applications of non-invasive brain stimulation in neurological and psychiatric diseases.

Introduction

Motor rehabilitation after cerebral injury such as stroke depends on neural plasticity, including synaptic strengthening by long-term potentiation (LTP) [1–4]. Paired associative stimulation (PAS_{LTP}) of the human primary motor cortex (M1) can induce an increase in corticospinal excitability as measured by motor evoked potentials (MEPs) beyond the stimulation period [5],

which resembles LTP as studied at the cellular level [6–9]. However, LTP-*like* plasticity induced by PAS_{LTP} is regulated by homeostatic metaplasticity [6, 10, 11], i.e., a higher-order form of plasticity, which keeps neuronal and network excitability in a physiological range [12–15]. This homeostatic regulation implies that repeated induction of LTP-*like* plasticity at short delays is suppressed, which may limit the therapeutic potential of PAS_{LTP} to increase corticospinal excitability.

Experiments in animals, however, show that the brain possesses powerful mechanisms, which permit continued synaptic strengthening in the context of prior LTP. One such mechanism is based on an N-methyl-D-aspartate receptor (NMDAR)-dependent form of metaplasticity by which continued synaptic strengthening is possible through activation of metabotropic glutamate receptors (mGluRs) [16]. In addition, inhibition of glycogen synthase kinase-3 beta (GSK3β) results in a ~60 min lasting blockade of subsequent induction of NMDAR-dependent long-term depression (LTD), because expression of LTD requires a high level of GSK3β activity [17]. These mechanisms ensure that information encoded by LTP is not erased during ongoing neural activity, but can be retained. On the system level of the human cortex it has been shown, that motor learning immediately following PAS_{LTP} is not suppressed, as would be expected in the framework of homeostatic metaplasticity, but rather facilitated [18, 19]. In addition, several other studies have occasionally reported non-homeostatic metaplasticity between two consecutive non-invasive brain stimulation protocols, mainly at short intervals of 30min or less [20–24], for review [25]. Even though these studies provide system-level evidence for non-homeostatic interactions between plasticity-inducing non-invasive brain stimulation protocols, and motor learning, respectively, the conditions favoring non-homeostatic vs. homeostatic metaplasticity in the human brain remain poorly understood.

The present study investigated the role of time between two consecutive PAS_{LTP} protocols for repetitive induction of LTP-*like* plasticity in M1 of healthy human subjects. We studied the interactions between two identical sessions of PAS_{LTP} spaced at inter-PAS_{LTP} intervals (IPI) of 10, 30, 60 and 180min. Findings show that metaplasticity in human M1 is expressed in a time-dependent manner with a window of non-homeostatic metaplasticity at an IPI of 30min. If spaced by 30min, two consecutive PAS_{LTP} sessions can augment LTP-*like* plasticity in human M1. These findings may inspire further research on optimized therapeutic applications of non-invasive brain stimulation techniques in a clinical setting.

Materials and Methods

Subjects

Written informed consent was obtained from all subjects prior to participation. None of the subjects had a history of neurological or psychiatric disease or was on CNS-active drugs at the time of the experiments and all subjects were checked for contraindications to transcranial magnetic stimulation (TMS) [26]. The study conformed to the Declaration of Helsinki and was approved by the ethics committee of the Medical Faculty of Goethe-University Frankfurt. Twenty-seven subjects were screened for a significant PAS_{LTP}-induced increase in motor evoked potential (MEP) amplitude ≥ 1.1 (ratio of mean MEP amplitude post-PAS_{LTP} / pre-PAS_{LTP}) [27, 28]. Sixteen subjects fulfilled this criterion (PAS_{LTP} responders) and were included into the study. Of those, four subjects withdrew consent after the first experiment for experiencing some discomfort by TMS. Thus, complete datasets were obtained in 12 subjects (6 females, mean (± SEM) age, 25.6 ± 1.4 years). All subjects were right-handed according to the Edinburgh handedness questionnaire [29].

Electromyography (EMG) recordings

Surface EMG recordings were obtained from the right abductor pollicis brevis (APB) muscle using Ag-AgCl electrodes in a belly-tendon montage. The raw EMG signal was amplified and band-pass filtered (20Hz–2kHz, Counterpoint Mk2 electromyograph, Dantec, Denmark), digitized at an A/D rate of 5kHz (Micro1401, Cambridge Electronic Design, UK) and stored in a laboratory computer for online display and offline analysis, using customized software (Spike2 for Windows, Version 3.05, Cambridge Electronic Design).

Transcranial magnetic stimulation (TMS)

Subjects were seated comfortably in a reclining chair. TMS was performed using a Magstim 200 magnetic stimulator (Magstim Company, UK) with a monophasic current waveform. Stimuli were applied to the hand area of left M1 through a figure-of-eight coil (inner diameter of each wing, 70mm) with the handle pointing backwards and 45° away from midline. The optimal coil position for eliciting MEPs in the right APB was marked with a soft-tipped pen on the scalp in order to ensure constant placement of the coil throughout the experiment.

At the beginning of each experiment the resting motor threshold (RMT) was measured, which was defined as the lowest intensity (indicated in percent of maximum stimulator output, MSO) that elicited small MEPs (>50 μV) in at least five out of ten consecutive trials in the resting APB. RMT was determined to nearest 1% of MSO. Thereafter, the intensity to elicit MEPs of, on average, 1mV peak-to-peak amplitude (SI_{1mV}) was determined in the resting APB. This stimulus intensity was then kept constant throughout a given session of the main experiment (Fig 1).

Paired associative stimulation (PAS_{LTP})

PAS_{LTP} was applied according to a protocol originally described by Stefan and colleagues [5] and slightly modified by our group [6, 10]. Briefly, electrical stimulation of the median nerve at

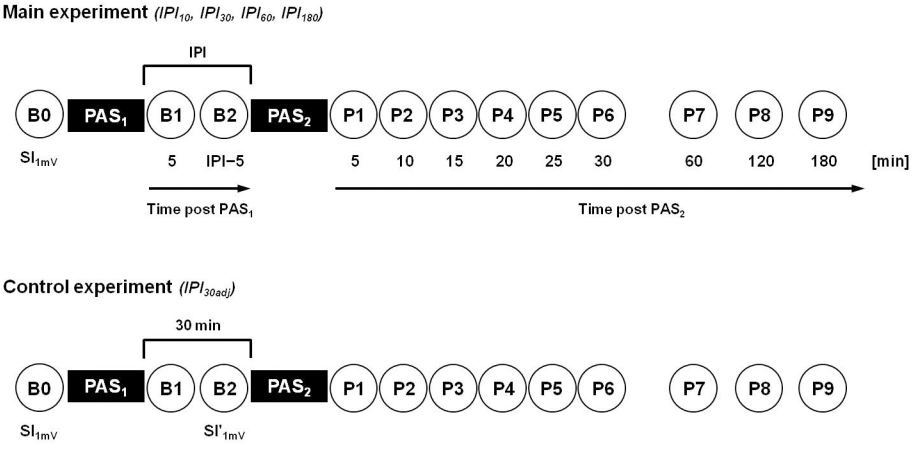

Fig 1. Time line of experiments. In the main experiment, motor-evoked potentials (MEPs) were recorded at all time points (circles; B0, B1-B2, P1-P9) with SI_{1mV}, i.e. the stimulation intensity that evoked MEPs of, on average, 1mV peak-to-peak amplitude in the resting *abductor pollicis brevis* muscle at baseline (time point B0). Note that subjects took part in four experimental sessions in a crossover design with different intervals between the consecutive sessions of two identical LTP-*like* plasticity inducing PAS_{LTP} protocols (PAS_1, PAS_2): 10min (IPI_{10}), 30min (IPI_{30}), 60min (IPI_{60}), and 180min (IPI_{180}). In the control experiment (IPI_{30adj}) SI_{1mV} was readjusted at time point B2 to match baseline MEPs of 1mV peak-to-peak amplitude (SI'_{1mV}). Otherwise, the control experiment was identical to the IPI_{30} condition of the main experiment.

doi:10.1371/journal.pone.0131020.g001

the wrist of the right hand was applied through a bipolar electrode (cathode proximal), using constant-current square-wave pulses (duration, 1ms) at an intensity of three times the perceptual sensory threshold. Each stimulus was followed by single-pulse TMS of the left M1 at SI_{1mV}. The interstimulus interval equaled the individual N20-latency of the median nerve somatosensory-evoked cortical potential plus 2ms (mean ± SEM, 21.3 ± 0.3ms). At this or similar intervals PAS induces an LTP-*like* increase of MEPs in the majority of subjects [5, 6, 27, 30–32]. PAS_{LTP} consisted of 225 stimulus pairs applied at a frequency of 0.25Hz. The level of attention, a significant modulator of PAS_{LTP} effects [33], was controlled and attention was maximized to the stimulated hand by a light emitting diode (LED) attached to the right wrist which flashed randomly (0.2–1Hz) during PAS_{LTP}. Subjects were requested to count and report the total number of flashes as correctly as possible at the end of PAS_{LTP}.

Experimental design

In the main experiment, all subjects took part in four different sessions in a pseudorandomized crossover design (Fig 1). Each session consisted of two identical, consecutive PAS_{LTP} protocols (PAS_1, PAS_2) with a specific inter-PAS_{LTP} interval (IPI). Intervals were 10min (IPI_{10}), 30min (IPI_{30}), 60min (IPI_{60}) and 180min (IPI_{180}). Between PAS_1 and PAS_2 subjects were requested to stay awake, keep seated and not to use their stimulated hand. The order of IPI conditions was pseudo-randomized across subjects and experimental sessions were separated by at least three days to avoid carry-over effects (mean individual minimum inter-session interval 7.6 days, range 3–14 days).

To test whether the significant PAS_2-induced increase of MEP amplitude in the IPI_{30} condition could be attributed to the PAS_1-induced increase in MEP amplitude at time point B2 immediately before PAS_2 (see below and cf. **Figs** 2 and 3), we conducted a control experiment (IPI_{30adj}), in which we readjusted MEP amplitudes at time point B2 by reducing the stimulation intensity (SI'_{1mV}) in order to match baseline MEPs of, on average, 1mV peak-to-peak amplitude. The readjusted stimulation intensity SI'_{1mV} was then used for PAS_2 and all following MEP measurements. Otherwise the control experiment was identical to the IPI_{30} condition of the main experiment (Fig 1). Nine subjects (4 females, mean (± SEM) age, 26.1 ± 1.6 years) took part in the control experiment.

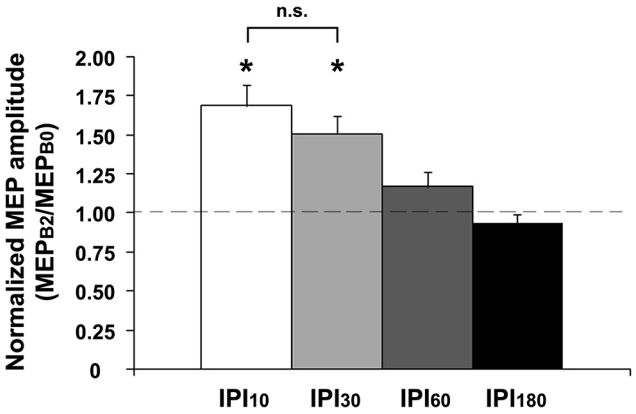

Fig 2. PAS_1-induced increases in MEP amplitude. PAS_1 resulted in comparable immediate MEP amplitude increases (MEP_{B1}/MEP_{B0}) in all IPI conditions (data not shown). At time point B2 (MEP_{B2}/MEP_{B0}), MEP amplitude increases were present 10min (IPI_{10}) and 30min (IPI_{30}) after PAS_1, but no longer at 60min (IPI_{60}) and 180min (IPI_{180}) after PAS_1. Asterisks indicate significant differences from 1 ($P < 0.01$; one-sample two-tailed *t* tests). Note that MEP_{B2}/MEP_{B0} was not significantly different between conditions IPI_{10} and IPI_{30} ($P > 0.09$; paired two-tailed *t* tests). Data are means ± 1 SEM from twelve subjects.

doi:10.1371/journal.pone.0131020.g002

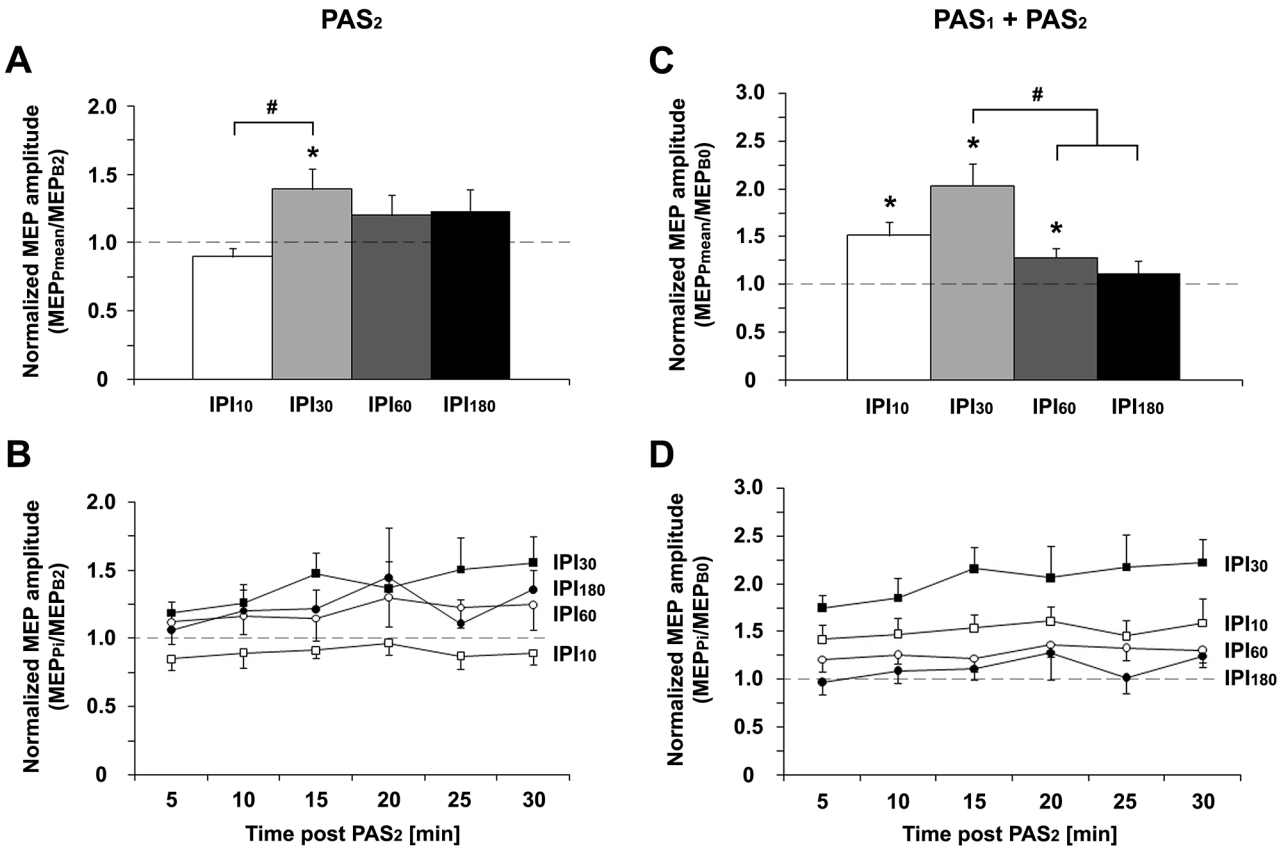

Fig 3. PAS_2-induced increases in MEP amplitude after PAS_1-priming. A: The PAS_2-induced increase in MEP amplitude after PAS_1-priming (MEP_{Pmean}/MEP_{B2}) was significantly higher at IPI_{30} than IPI_{10} (#, $P < 0.05$; paired two-tailed *t* test). **B**: Time course of MEP_{Pi}/MEP_{B2} (i = 1,2,. . .,6) over 30min after PAS_2 for IPI_{10} (open squares), IPI_{30} (filled squares), IPI_{60} (open circles), and IPI_{180} (filled circles). **C**: The cumulative effect of PAS_1 and PAS_2 on MEP amplitudes (MEP_{Pmean}/MEP_{B0}) was significantly higher for IPI_{30} than for IPI_{60} and IPI_{180} (#, $P < 0.05$; paired two-tailed *t* tests), and showed a trend to be higher for IPI_{30} vs. IPI_{10} ($P = 0.095$; paired two-tailed *t* test). **D**: Time course of MEP_{Pi}/MEP_{B2} (i = 1,2,. . .,6) over 30min after PAS_2 for IPI_{10} (open squares), IPI_{30} (filled squares), IPI_{60} (open circles), and IPI_{180} (filled circles). *, $P < 0.05$; one-sample two-tailed *t* tests. Data are means ± 1 SEM from twelve subjects.

doi:10.1371/journal.pone.0131020.g003

Quantification of PAS_{LTP} effects

The PAS_1 effect was quantified by comparing 20 single-trial peak-to-peak MEP amplitudes at SI_{1mV} 5min after PAS_1 (time point B1) with those at baseline immediately before PAS_1 (time point B0; **cf.** Fig 1). The inter-trial interval was 10s ± 25% to limit anticipation of the next trial. If the PAS_1 effect (ratio MEP_{B1}/MEP_{B0}) was < 1.1, the experiment was stopped, the data were discarded and the experiment was repeated on another day, to minimize intra-individual variability [34] and ensure PAS_1-induced LTP-*like* plasticity in all experiments (overall, 12 repeated experiments in all subjects; 1.0 ± 0.4 repeated experiments per subject). Five minutes before the second PAS_{LTP} intervention (PAS_2) another block of 20 MEPs was measured (time point B2; in case of IPI_{10} B1 was taken as B2). After PAS_2 six blocks of MEP measurements were performed at intervals of 5min (time points P1–P6) to monitor PAS_2 effects on MEPs for 30min after the end of PAS_2. In six subjects, additional MEP measurements were conducted 60min (time point P7), 120min (P8), and 180min (P9) after PAS_2 to determine the time course of the return of PAS_2-induced MEP amplitude increases (see Results) back to baseline. All measurements were recorded in the resting APB at SI_{1mV}, except for the control experiment (IPI_{30adj}), in which the TMS intensity was readjusted to SI'_{1mV} at time point B2 and then used for PAS_2 and all following MEP measurements. Complete voluntary relaxation was monitored

by audio-visual feedback of the EMG raw signal at high gain (at 50μV / division). Trials contaminated with voluntary EMG activity were excluded from analysis (<1% of all trials).

Statistical analyses

Statistical testing was performed with IBM SPSS Statistics (Version 20.0.0). To test for differences of RMT, SI_{1mV}, and MEP amplitude at baseline (time point B0) independent one-way repeated measures analyses of variance (rmANOVAs) with the within-subject factor IPI (IPI_{10}, IPI_{30}, IPI_{60}, IPI_{180}) were conducted. To test for differences of the PAS_1 effect one-way rmANOVAs with the within-subject factor IPI (IPI_{10}, IPI_{30}, IPI_{60}, IPI_{180}) were performed separately on mean MEP amplitudes at time points B1 and B2, respectively, normalized to the individual mean MEP amplitude at time point B0 (MEP_{B1}/MEP_{B0}; MEP_{B2}/MEP_{B0}). To test for the effects of IPI (IPI_{10}, IPI_{30}, IPI_{60}, IPI_{180}) and TIME (P1–P6) on PAS_2 effects, two-way rmANOVAs were performed separately on mean MEP amplitudes at time points P1–P6 normalized to the individual mean MEP amplitude at time points B0 and B2, respectively (MEP_{Pi}/MEP_{B0}, MEP_{Pi}/MEP_{B2}, i = 1,2,. . .,6). Normalization to B2 provides information specifically on the effects of PAS_2 while normalization to B0 provides information on the cumulative effects of PAS_1 and PAS_2.

Mauchly's test was applied to test for non-sphericity and in case of violation of sphericity the Greenhouse-Geisser correction was used. Conditional on significant main effects or their interactions in the rmANOVAs, *post hoc* pairwise comparisons or one-sample two-tailed *t* tests were performed. The Bonferroni correction was applied to adjust for multiple comparisons.

To compare the time course of PAS_1- and PAS_2-induced changes in MEP amplitude, the MEP amplitude raw data after PAS_1 and PAS_2 were fitted independently with a linear mixed effects model with fractional polynomials at all IPIs. PAS_1 data comprised mean MEP amplitudes measured at time points B0 (time: 0min) and B2 across IPI conditions (time: 10min at IPI_{10}, 30min at IPI_{30}, 60min at IPI_{60}, and 180min at IPI_{180}), whereas PAS_2 data comprised mean MEP amplitudes measured at time points B2 (time: 0min), P2 (time: 10min), P6 (time: 30min), P7 (time: 60min), and P9 (time: 180min), respectively, for each IPI condition separately. Model functions for PAS_1- and PAS_2 effects on MEP amplitudes were compared using *F*- and *t*-statistics, respectively.

In all tests the significance level was set to $P < 0.05$. All data are expressed as means ± SEM.

Results

All subjects tolerated the experimental procedures well without any adverse effects.

Baseline excitability data (RMT, SI_{1mV}, MEP)

The data are summarized in Table 1. There were no significant differences between IPI conditions on RMT ($F_{3,33} = 0.55$, $P = 0.53$), SI_{1mV} ($F_{3,33} = 0.46$, $P = 0.71$), or MEP amplitude ($F_{3,33} = 1.07$, $P = 0.38$) at baseline (time point B0).

PAS_1 effects on MEP amplitudes (comparison of time points B1 and B2 vs. B0)

The one-way rmANOVA for the PAS_1 effect at time point B1 (MEP_{B1}/MEP_{B0}) showed no significant difference between IPI conditions ($F_{3,33} = 0.71$, $P = 0.55$), indicating similar PAS_1-induced LTP-*like* plasticity across all IPI conditions. MEP_{B1}/MEP_{B0} was > 1.0 for IPI_{10} (1.69 ± 0.13; $t = 5.25$, $P = 0.0003$), IPI_{30} (1.49 ± 0.10; $t = 5.01$, $P = 0.0004$), IPI_{60} (1.50 ± 0.07;

Table 1. Summary of baseline excitability measures in the different IPI conditions.

Condition	RMT	SI_{1mV}	MEP
IPI_{10}	40.1 ± 2.1	50.8 ± 2.2	0.97 ± 0.04
IPI_{30}	39.3 ± 1.6	51.4 ± 2.0	0.91 ± 0.05
IPI_{60}	38.9 ± 1.8	50.5 ± 2.4	0.93 ± 0.04
IPI_{180}	38.8 ± 1.4	49.6 ± 1.7	1.02 ± 0.05

Abbreviations: IPI, inter-PAS_{LTP} interval [in min, index]; MEP, motor-evoked potential [in mV]; RMT, resting motor threshold [in %maximum stimulator output, MSO]; SI_{1mV}, stimulation intensity that induces MEPs of 1mV peak-to-peak amplitude on average [in %MSO].

doi:10.1371/journal.pone.0131020.t001

$t = 7.57$, $P < 0.0001$), and IPI_{180} (1.53 ± 0.13; $t = 4.12$, $P = 0.0017$). In contrast, the one-way rmANOVA for the PAS_1 effect at time point B2 (MEP_{B2}/MEP_{B0}) immediately before PAS_2 showed a significant effect of IPI ($F_{3,33} = 11.74$, $P < 0.001$). MEP_{B2}/MEP_{B0} was > 1.0 for IPI_{10} (1.69 ± 0.13; $t = 5.25$, $P = 0.0003$) and IPI_{30} (1.51 ± 0.12; $t = 4.32$, $P = 0.0012$), but no longer for IPI_{60} (1.17 ± 0.09; $t = 1.90$, $P = 0.084$) or IPI_{180} (0.94 ± 0.05; $t = -1.20$, $P = 0.26$) (Fig 2). Of note, there was no significant difference of MEP_{B2}/MEP_{B0} between conditions IPI_{10} and IPI_{30} ($P > 0.9$), indicating similar persistence of PAS_1-induced LTP-*like* plasticity in these conditions at time point B2 immediately before PAS_2.

PAS_2 effects on MEP amplitudes (comparison of time points P1-P6 vs. B2)

The two-way rmANOVA for the PAS_2 effect on MEP amplitudes at time points P1–P6 (MEP_{Pi}/MEP_{B2}, i = 1,2,. . .,6) showed a significant effect of IPI ($F_{3,33} = 2.94$, $P = 0.048$) and TIME ($F_{5,55} = 2.50$, $P = 0.041$), whereas the interaction IPI with TIME was not significant ($F_{15,165} = 0.73$, $P = 0.75$) (Fig 3A and 3B). MEP_{Pmean}/MEP_{B2} was > 1.0 for IPI_{30} (1.39 ± 0.15; $t = 2.59$, $P = 0.025$), but not for IPI_{10} (0.90 ± 0.06; $t = -1.72$, $P = 0.11$), IPI_{60} (1.20 ± 0.15; $t = 1.34$, $P = 0.21$) or IPI_{180} (1.23 ± 0.16; $t = 1.48$, $P = 0.17$). *Post hoc* testing showed that MEP amplitudes after PAS_2 (MEP_{Pmean}/MEP_{B2}) were significantly different between conditions IPI_{10} and IPI_{30} ($P = 0.047$), while all other pairwise comparisons were not significant (all $P > 0.2$).

It could be argued that the significant PAS_2 effect on MEP amplitudes at IPI_{30} was due to the increased MEP amplitude at time point B2 immediately before PAS_2 (cf. Fig 2). To address this issue, we conducted a control experiment (IPI_{30adj}), in which we readjusted MEP amplitudes at time point B2 immediately before PAS_2 by reducing the stimulation intensity (SI'_{1mV}) in order to match baseline MEPs (0.99 ± 0.05mV at time point B0 with SI_{1mV} = 45.8 ± 1.6% MSO; 1.01 ± 0.06mV at time point B2 with SI'_{1mV} = 44.2 ± 1.6%MSO, $t = -0.29$, $P > 0.7$; paired two-tailed *t* test). Thus, whilst PAS_{LTP} induced similar MEP increases at time point B1 (MEP_{B1}/MEP_{B0}) in the control (IPI_{30adj}, 1.31 ± 0.05; $t = 6.62$, $P = 0.0002$, one-sample two-tailed *t* test) and the main experiment (IPI_{30}, 1.49 ± 0.10; $t = 5.01$, $P = 0.0004$, one-sample two-tailed *t* test; IPI_{30adj} vs. $IPI_{30:}$ $P > 0.15$, unpaired two-tailed *t* test), MEP_{B2}/MEP_{B0} was significantly different between the two experiments (IPI_{30adj}: 1.02 ± 0.04, $t = 0.38$, $P > 0.7$; IPI_{30}: 1.51 ± 0.12; $t = 4.32$, $P = 0.0012$, one-sample two-tailed *t* tests; IPI_{30adj} vs. $IPI_{30:}$ $P = 0.0025$, unpaired two-tailed *t* test; Fig 4). However, PAS_2 induced a similar increase in MEP amplitudes (MEP_{Pmean}/MEP_{B2}) in the control (IPI_{30adj}: 1.29 ± 0.09, $t = 3.09$, $P = 0.015$) compared to the main experiment (IPI_{30}: 1.39 ± 0.15; $t = 2.59$, $P = 0.025$; one-sample two-tailed *t* tests; IPI_{30adj} vs. $IPI_{30:}$ $P > 0.5$, unpaired two-tailed *t* test; Fig 4). This finding strongly suggested that the increased MEP amplitude at time point B2 at IPI_{30} was not relevant for the significant PAS_2-induced

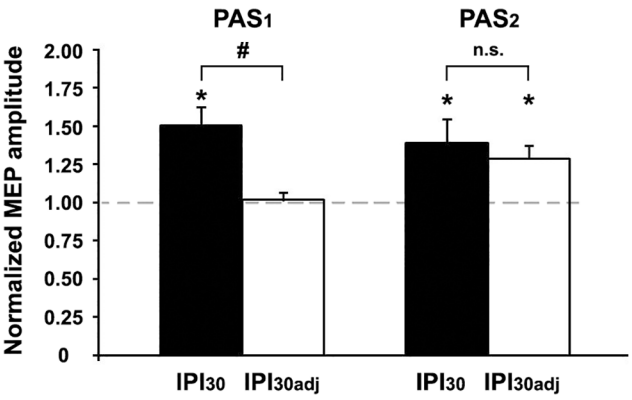

Fig 4. PAS_2-induced increase in MEP amplitude after PAS_1-priming in the control experiment (IPI_{30adj}). MEP amplitudes at time point B2 immediately before PAS_2 were successfully readjusted by reducing the stimulation intensity (SI'_{1mV}) to match baseline MEPs at time point B0 (PAS_1, $MEP_{B2/B0}$). Despite this readjustment, PAS_2 induced a similar increase in MEP amplitudes in the control (IPI_{30adj}) compared to the main experiment (IPI_{30}) (PAS_2, MEP_{Pmean}/MEP_{B2}). *, $P < 0.05$, one-sample two-tailed *t* tests; #, $P < 0.01$, unpaired two-tailed *t* test. Data from the control experiment are from nine subjects, means ± 1 SEM.

doi:10.1371/journal.pone.0131020.g004

increase of MEP amplitudes in this condition. This notion was further supported by the observation, that PAS_2 had significantly different effects on MEP amplitudes at IPI_{30} vs. IPI_{10} in the main experiment (cf. Fig 3A and 3B), although MEP amplitudes were increased to a similar level immediately before PAS_2 (MEP_{B2}/MEP_{B0}) in these two conditions (cf. Fig 2).

Cumulative PAS_1 and PAS_2 effects on MEP amplitudes (comparison of time points P1-P6 vs. B0)

The two-way rmANOVA for the cumulative PAS_1 and PAS_2 effect on MEP amplitudes at time points P1–P6 (MEP_{Pi}/MEP_{B0}, i = 1,2,. . .,6) showed a significant effect of IPI ($F_{3,33} = 11.36$, $P < 0.0001$) and TIME ($F_{5,55} = 2.49$, $P = 0.042$), whereas the interaction IPI with TIME was not significant ($F_{15,165} = 0.66$, $P = 0.82$) (Fig 3C and 3D). MEP_{Pmean}/MEP_{B0} was > 1.0 for IPI_{10} (1.51 ± 0.14; $t = 3.61$, $P = 0.0041$), IPI_{30} (2.04 ± 0.23; $t = 4.54$, $P = 0.0009$), and IPI_{60} (1.28 ± 0.09; $t = 2.98$, $P = 0.013$), but not for IPI_{180} (1.12 ± 0.12; $t = 0.97$, $P = 0.35$). *Post hoc* testing showed significantly higher MEP amplitudes after PAS_2 (MEP_{Pmean}/MEP_{B0}) for IPI_{30} than for IPI_{60} ($P = 0.009$) and IPI_{180} ($P = 0.01$), and a trend for higher MEP amplitudes for IPI_{30} vs. IPI_{10} ($P = 0.095$). Notably, at IPI_{30} the MEP amplitude increase 30 minutes after PAS_2 normalized to baseline (MEP_{P6}/MEP_{B0} = 2.22 ± 0.25; Fig 3D) was significantly higher than the MEP amplitude increase 30 minutes after PAS_1 in this condition (MEP_{B2}/MEP_{B0} = 1.51 ± 0.12; $P = 0.018$, paired two-tailed *t* test), indicating that consecutive application of PAS_1 and PAS_2 at IPI_{30} induced significantly higher MEP increases than application of PAS_1 alone.

Modelling of PAS_1 and PAS_2 effects on MEP amplitudes

Computational modelling of the PAS_1 and PAS_2 effects on the absolute MEP amplitude raw data as a function of time revealed highly significant differences between PAS_1 and PAS_2 MEP functions ($P < 0.0001$) (Fig 5). *Post hoc* testing showed significant differences between the PAS_1 and PAS_2 model functions for all IPI conditions ($P < 0.0001$ each), suggesting that PAS_2 effects on MEP amplitudes were modulated by prior application of PAS_1 in all IPI conditions. Specifically, at IPI_{10} PAS_2 prolonged the MEP increase induced by PAS_1. At IPI_{30}, PAS_2 induced an extra MEP increase, notably with a longer time course as compared to the PAS_1-

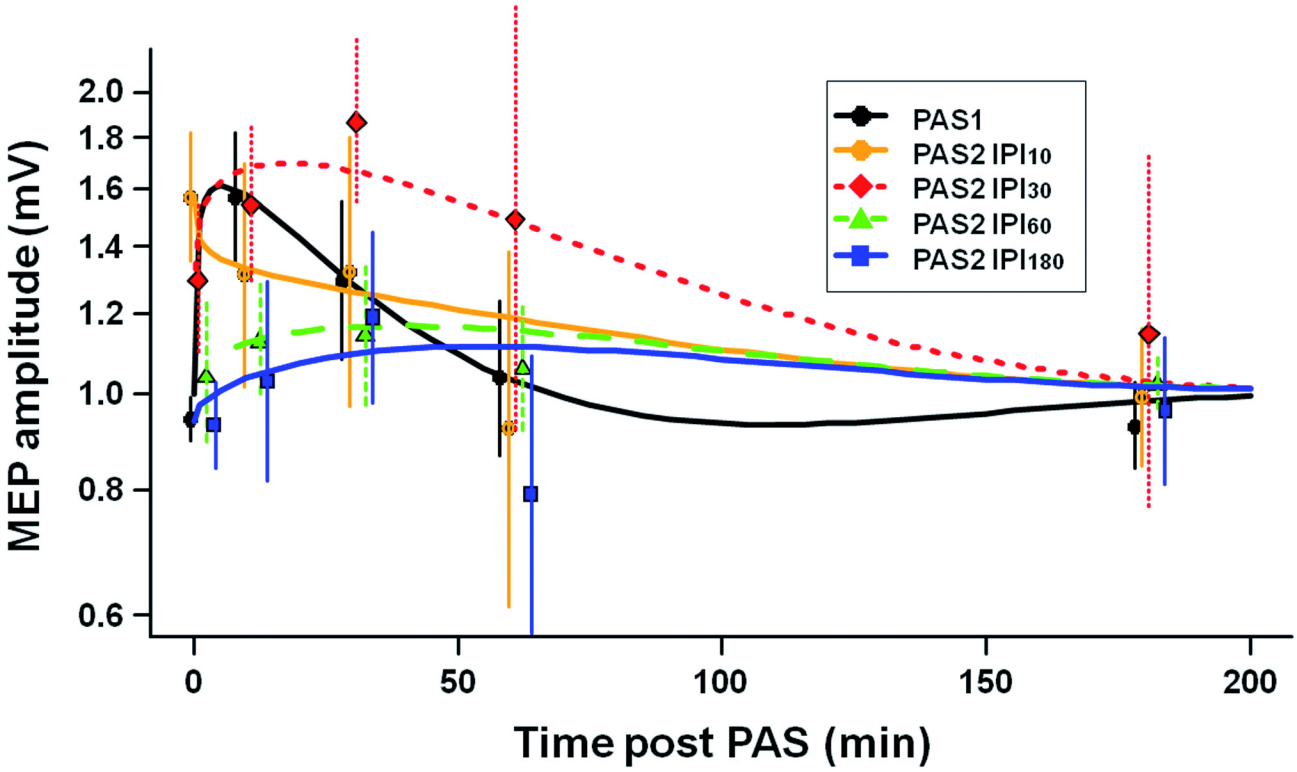

Fig 5. Computational modeling of the time course of PAS_1 and PAS_2 effects. MEP amplitudes as a function of time modeled from the experimental MEP raw data independently for PAS_1 and PAS_2 at all IPIs. PAS_1 and PAS_2 model functions, i.e. the time course of MEP amplitude changes after PAS_1 vs. those after PAS_2, were significantly different at all IPIs ($P < 0.0001$ each). In addition, PAS_2 effects at IPI_{10} and IPI_{30} were significantly different from PAS_2 effects at IPI_{60} and IPI_{180} ($P < 0.0001$), and PAS_2 effects at IPI_{10} from PAS_2 effects at IPI_{30} ($P = 0.033$), but not PAS_2 effects at IPI_{60} from those at IPI_{180} ($P > 0.5$). Experimental data are shown as mean ± SEM. Note that data for PAS_2 at time points 60min and 180min post PAS_2 are from six subjects only, whereas all other data are from twelve subjects (see Material and Methods). Y-axis, logarithmic scaling.

doi:10.1371/journal.pone.0131020.g005

induced MEP increase. In contrast, at IPI_{60} and IPI_{180} there was no significant change in MEP amplitudes after PAS_2.

In addition, the time courses of PAS_2-induced MEP amplitude changes were significantly different between IPI conditions ($P < 0.0001$). *Post hoc* analysis showed that PAS_2 effects at IPI_{10} and IPI_{30} were significantly different from PAS_2 effects at IPI_{60} and IPI_{180} (all $P < 0.0001$), and PAS_2 effects at IPI_{10} were significantly different from PAS_2 effects at IPI_{30} ($P = 0.033$), whereas the comparison of PAS_2 effects at IPI_{60} and IPI_{180} showed no significant differences ($P > 0.5$). These differences are explained by a prolonged MEP increase at IPI_{30} compared to all other conditions (Fig 5).

Discussion

We showed here that LTP-*like* plasticity can be augmented in human M1 when two consecutive PAS_{LTP} protocols are spaced by 30min. In contrast, at longer intervals (60-180min) we found a suppressive interaction between two consecutive PAS_{LTP} protocols. These findings support the notion of non-homeostatic and homeostatic metaplasticity, respectively, and will be discussed in detail below.

The PAS_1-induced MEP increase lasted for 30-60min, in accord with the literature, and represents a form of plasticity resembling LTP as studied at the cellular level [5, 7, 9, 35]. In contrast, the effects of PAS_2 on MEP amplitude depended critically on the interval to PAS_1.

Computational modelling of the time courses of MEP amplitude (Fig 5) revealed significant differences between PAS_1 and PAS_2 effects on MEP amplitude for all IPI conditions, indicating that priming M1 by PAS_1 modulated the effects of a subsequent identical PAS_2 protocol for at least three hours.

Metaplasticity constitutes a higher-order form of plasticity, which regulates the magnitude and duration of synaptic plasticity in an activity-dependent manner [12]. Importantly, it modulates the plasticity state of neurons and networks, i.e. the induction of LTP subsequent to the priming stimulation, in the absence of synaptic plasticity induced by the priming stimulation itself. Here, we demonstrated a homeostatic interaction between PAS_1 and PAS_2 at IPI_{60} and IPI_{180} (Fig 3) in the absence of any persistent PAS_1-induced MEP increase at the time of PAS_2 (Fig 2). Further, both magnitude and/or duration of PAS_2-induced LTP-*like* plasticity were modulated by PAS_1 (Fig 5). Thus, these findings support the notion that the interactions between PAS_1 and PAS_2 effects described in the present study represent a form of metaplasticity similar to the ones reported at the cellular level [12, 36].

Previous studies have reported on metaplasticity between two consecutive identical non-invasive brain stimulation protocols in the human M1 [10, 20–24, 37–40]. The predominant interaction was homeostatic metaplasticity, but several occasions of non-homeostatic metaplasticity have also been reported, predominantly at short IPIs of 3-20min (for review, [25]). This non-homeostatic metaplasticity resulted in *late* LTD- and LTP-*like* changes in corticospinal excitability that were prevented by pharmacological blockade of NMDA receptors by dextromethorphan [24] and that were resistant to de-depression interventions such as voluntary contraction of the target muscle [22]. These data are compatible with the finding in the present study that two consecutive PAS_{LTP} protocols, if spaced by 30min, resulted in non-homeostatic augmentation of LTP-*like* plasticity with a prolonged duration.

These time-dependent interactions between PAS_1 and PAS_2 may be explained in the framework of a cascade model of synaptic plasticity [41]. As synapses are modified during the course of LTP, they change between *discrete* mechanistic states [42–44]. For example, silent synapses, i.e. synapses containing NMDARs, but being devoid of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) move to a "recently silent state" (i.e. with inserted AMPARs), in which they initially cannot be further potentiated. Additional potentiation is possible only if they move to the "active state", which occurs at around 30min after LTP induction [42, 45]. Recent results from organotypic entorhino-hippocampal slice cultures suggest that repetitive magnetic stimulation indeed may lead to an activation of silent synapses [46]. As PAS_1 and PAS_2 were identical in our study and thus presumably modified the same set of synapses, such a cascade model of synaptic plasticity states may explain why PAS_2 induced significant LTP-*like* plasticity at IPI_{30}, but not at IPI_{10}. In addition, the MEP increase after PAS_2 at IPI_{30} may have been enabled by LTP-induced suppression of subsequent LTD. In rat hippocampus NMDAR-dependent LTP inhibits GSK3β, resulting in a ~60 minutes lasting blockade of subsequent NMDAR-dependent LTD induction [17]. In contrast, the suppressive interactions between PAS_1 and PAS_2 at IPI_{60} and IPI_{180} are in line with homeostatic metaplasticity [12]. Evidence in support of the notion of a delayed onset of homeostatic metaplasticity comes from animal experiments that showed that an experience-dependent increase in the NR2A/NR2B NMDAR subunit ratio, which is associated with a rightward shift of the synaptic modification threshold that favors induction of LTD over LTP [47, 48], did not occur within the first ~60min after a priming protocol [49].

An alternative explanation for the augmenting PAS_2 effect on MEPs at IPI_{30} may have been gating [50] by the increased level of cortical excitability immediately prior to PAS_2 in this condition (time point B2, Fig 2). However, this explanation is rather unlikely, as (i) computational modelling of MEP time functions after PAS_2 showed significant differences between conditions

IPI_{10} and IPI_{30} (cf. Fig 5), although the PAS_1-induced MEP increases immediately before PAS_2 were comparable in these two conditions (cf. Fig 2), and (ii) a control experiment (IPI_{30adj}), in which we readjusted MEP amplitudes after PAS_1 immediately before PAS_2 to match baseline values, found similar PAS_2 effects as in the main experiment (IPI_{30}), in which MEP amplitudes were not readjusted (Fig 4).

At IPI_{30}, PAS_2 induced significant MEP increases over and above those induced by PAS_1. The cumulative PAS_1 and PAS_2 increase of MEP amplitude at IPI_{30} significantly exceeded the PAS_1 effect alone, and the cumulative effects of PAS_1 and PAS_2 at IPI_{60} and IPI_{180}. These results show that LTP-*like* plasticity can be augmented in human M1, if consecutive PAS_{LTP} protocols are properly spaced within a window of non-homeostatic metaplasticity.

In contrast, homeostatic metaplasticity, i.e. the suppressive interaction between consecutive PAS_{LTP} protocols at longer intervals (IPI_{60} and IPI_{180}), may prevent runaway LTP, enabling the human motor network to maintain its modifiability within a useful dynamic range. In line with this notion, dysfunctional homeostatic metaplasticity can result in a deficient control of activity-dependent plasticity, as seems to be the case in task-dependent dystonia [51, 52]. The mechanisms of homeostatic metaplasticity at IPI_{60} and IPI_{180} are as of yet unclear, but may involve Ca^{2+} influx through voltage-gated Ca^{2+} channels [53], or intracellular Ca^{2+} stores [54].

Studies in experimental animals have provided evidence, that an mGluR-dependent form of LTP is retained in the context of NMDAR-dependent increased synaptic strength [16, 55]. Thus, continued experience leads to increased synaptic strength over time, although NMDAR-dependent LTP is occluded during continued neural activity. It could be speculated that LTP-*like* plasticity induced by PAS_1 vs. PAS_2 at IPI_{30} in our experiments was due to different underlying physiological mechanisms, i.e. an NMDAR- and an mGluR-dependent form of LTP-*like* plasticity, respectively. This idea is indirectly supported by the following observations: (i) LTP-*like* plasticity induced by a single PAS_{LTP} protocol can be blocked by an NMDAR antagonist [7]; (ii) the PAS_1-induced LTP-*like* plasticity in the present study was likely saturated as the MEP amplitude increase of $\geq$1.5 is among the highest reported in the literature [30, 33, 56]; (iii) the time-course of PAS_1- vs. PAS_2-induced MEP changes at IPI_{30} in the present study was significantly different (cf. Fig 5). Thus, properly timed consecutive application of non-invasive brain stimulation protocols such as PAS_{LTP} may lead to augmentation of LTP-*like* plasticity through recruitment of different physiological mechanisms. Regardless of the underlying mechanisms, our results provide experimental evidence that consecutive applications of PAS_{LTP} can lead to significantly increased LTP-*like* plasticity as compared to a single PAS_{LTP} session.

At first sight, the present data are at variance with those from one previous study of our group where we found homeostatic metaplasticity between two consecutive PAS_{LTP} protocols if spaced by 30min [10]. However, the two studies differ in one important aspect: in this but not the previous study, we deliberately maximized LTP-*like* plasticity induced by PAS_{LTP} by including only those data with a PAS_1-induced MEP increase $\geq$1.1, and by ensuring high attention towards the stimulated hand, which is known to facilitate PAS_{LTP}-induced LTP-*like* plasticity [33]. As a result, the PAS_1–induced MEP increase in this study (time point B1, 1.49 ± 0.10, condition IPI_{30}) was significantly higher than in the previous study (1.14 ± 0.12; $t = 2.24$, $P = 0.036$; unpaired two-tailed t test). Previous work in mice showed that the expression of non-homeostatic metaplasticity critically depended on the level of the priming activity: only during strong but not weak stimuli, potentiated synapses could be further potentiated, specifically by induction of a switch in NMDAR and mGluR properties [16, 55]. Thus, the significantly stronger LTP-*like* plasticity induced by PAS_1 in this compared to our previous study [10] may explain why we observed augmentation of LTP-*like* plasticity in the present study only.

In summary, the present study demonstrated that LTP-*like* plasticity in the human M1 can be augmented by application of two consecutive identical PAS_{LTP} protocols if spaced by 30min, while homeostatic interaction occurred at intervals of 60-180min. These findings may inspire further research to optimize therapeutic applications of non-invasive brain stimulation in patients with neurological or psychiatric diseases to modify synaptic transmission in their disordered brain networks more effectively than hitherto possible.

Acknowledgments

We thank Prof. G.W. Thickbroom and Dr. P. Jedlicka for helpful comments on earlier versions of this manuscript.

Author Contributions

Conceived and designed the experiments: FM-D UZ. Performed the experiments: FM-D CL M-KL NA AF. Analyzed the data: FM-D EH. Wrote the paper: FM-D UZ.

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