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Human motor associative plasticity induced by paired bihemispheric stimulation

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Paired associative stimulation (PAS) is an effective non-invasive method to induce human motor plasticity by the repetitive pairing of peripheral nerve stimulation and transcranial magnetic stimulation (TMS) at the primary motor cortex (M1) with a specific time interval. Although the repetitive pairing of two types of afferent stimulation might be a biological basis of neural plasticity and memory, other types of paired stimulation of the human brain have rarely been studied. We hypothesized that the repetitive pairing of TMS and interhemispheric cortico-cortical projection or paired bihemispheric stimulation (PBS), in which the right and left M1 were serially stimulated with a time interval of 15 ms, would produce an associative long-term potentiation (LTP)-like effect. In this study, 23 right-handed healthy volunteers were subjected to a 0.1 Hz repetition of 180 pairings of bihemispheric TMS, and physiological and behavioural measures of the motor system were compared before, immediately after, 20 min after and 40 min after PBS intervention. The amplitude of the motor evoked potential (MEP) induced by the left M1 stimulation and its input–output function increased for up to ∼20 min post-PBS. Fine finger movements were also facilitated by PBS. Spinal excitability measured by the H-reflex was insensitive to PBS, suggesting a cortical mechanism. The associative LTP-like effect induced by PBS was timing dependent, occurring only when the interstimulus interval was 5–25 ms. These findings demonstrate that using PBS in PAS can induce motor cortical plasticity, and this approach might be applicable to the rehabilitation of patients with motor disorders.

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Abbreviations APs, action potentials; APB, abductor pollicis brevis; cRT, choice reaction time; EPSPs, excitatory postsynaptic potentials; FCR, flexor carpi radialis; H_{max}, maximum H-reflex amplitude; 9HPT, nine-hole peg test; I–O, input–output; IHI, interhemispheric inhibition; ISI, interstimulus interval; LTD, long-term depression; LTP, long-term potentiation; M1, primary motor cortex; MEP, motor evoked potential; M_{max}, maximum M-wave amplitude; PAS, paired associative stimulation; PBS, paired bihemispheric stimulation; rMT, resting motor threshold; RT, reaction time; SI, stimulus intensity; SI 1 mV, stimulus intensity required to produce a motor evoked potential of ∼1 V; SP, silent period; TMS, transcranial magnetic stimulation.

The human primary motor cortex (M1) has a great reorganization ability in adults following repetitions of simple movement and motor skill training (Karni *et al.* 1995; Pascual-Leone *et al.* 1995; Classen *et al.* 1998), environmental change (Muellbacher *et al.* 2000; Nilsson & Pekny, 2007) and injury to the sensorimotor system (Brasil-Neto *et al.* 1993; Chen *et al.* 2002; Nudo, 2003; Rossini *et al.* 2007). This maintained plasticity helps us to adjust to various external and internal conditions. Moreover, the plasticity in the motor cortex might be a physiological basis of motor learning and memory (Rioult-Pedotti *et al.* 2000; Ziemann *et al.* 2004). One intensively studied method to produce human M1 plasticity experimentally is paired associative stimulation (PAS), that is, the low-frequency repetitive pairing of electrical peripheral nerve stimulation and transcranial magnetic stimulation (TMS) over the M1 with a specific time interval (Stefan *et al.* 2000, 2002; Wolters *et al.* 2003; Ueki *et al.* 2006). Previous studies showed that the M1 plasticity induced by PAS is similar to associative long-term potentiation (LTP) or long-term depression (LTD). This LTP-like effect is likely to be Hebbian plasticity

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(Hebb, 1949), because it is lost or even reversed when TMS is applied immediately prior to the cortical arrival time of peripheral nerve stimulation.

However, it is not known whether other types of afferent stimulation of the M1 can produce associative plasticity when they are time-locked to the TMS over the M1. This scenario is considered likely to occur in humans if associative plasticity is a general principle of brain development and learning, as suggested by Hebb (1949).

One possible origin of afferent projection to the M1 is the contralateral M1. The human motor control of complex bimanual coordination is based on the intense functional connections within the bilateral M1. Lesion studies in patients have shown the functional relevance of transcallosal fibres for bimanual coordination (Serrien *et al.* 2001; Kennerley *et al.* 2002; Seitz *et al.* 2004). Although the transcallosal connections between M1 areas are more prominent in the proximal muscles of primates (Jenny, 1979; Pappas & Strick, 1981; Gould *et al.* 1986), several studies have suggested the existence of short inter-hemispheric conduction pathways for the hand M1 area, possibly through transcallosal fibres (Shibasaki *et al.* 1978; Wilkins *et al.* 1984; Brown *et al.* 1991; Hanajima *et al.* 2001). When paired TMS was applied to the right and left M1, the conditioning effect of the contralateral M1 stimulation began as early as 6 ms and reached a maximum at ∼10 ms (Ferbert *et al.* 1992; Gerloff *et al.* 1998).

Thus, we hypothesized that the repetitive pairing of TMS and interhemispheric cortico-cortical projection or paired bihemispheric stimulation (PBS) would produce an associative LTP-like effect. For the PBS, we utilized left M1 stimulation preceded by right M1 stimulation with a time interval of 15 ms, which can combine the inter-hemispheric projection from the right to left M1 and the TMS at the left M1. As the order of the paired inputs is important for effectively producing associative plasticity (Bi & Poo, 1998; Wolters *et al.* 2003, 2005), we decided to apply a delay of 15 ms, so that the inputs from the contralateral M1 would arrive at the targeted M1 area before the TMS. A previous animal study indicated that a time delay between two spikes of $<$20 ms could produce associative plasticity (Bi & Poo, 1998). We therefore also tested whether the effects of PBS were timing dependent by changing the interstimulus interval (ISI) at intervals of 10 ms from −25 ms to 25 ms.

In addition to the motor evoked potential (MEP) amplitudes, we also systematically examined the spinal motor neuronal excitability, the input–output (I–O) function of the M1 and various motor behaviours following PBS. We found PBS to be a promising method to induce associative plasticity in the human motor system (Koganemaru *et al.* 2008). A similar method to induce plastic change in order to increase cortico-spinal excitability was reported recently (Rizzo *et al.* 2009).

Methods

Subjects

The study was approved by the Committee of Medical Ethics of the Graduate School of Medicine, Kyoto University, Japan, and written informed consent was obtained from all participants. The experiments were performed with 23 healthy volunteers (22 men and 1 woman) aged 22–34 years (mean, 27.7 ± 4.6 years). None of the participants had a history of neurological illness or was taking medication. All of the volunteers were right handed, according to the Oldfield handedness inventory (Oldfield, 1971).

Recording procedures

The electromyographic activity was recorded from the right and left abductor pollicis brevis (APB) muscles, and the right and left flexor carpi radialis (FCR) muscles, using a pair of silver electrodes. The EMGs were amplified and filtered (bandpass, 5–2000 Hz), and digitized at a sampling rate of 10 kHz using the Neuroscan system (Neuroscan Co., Herndon, VA, USA).

Each subject was seated comfortably in an armchair. Focal TMS was performed using a flat figure-of-eight-shaped magnetic coil (outer diameter of each wing, 9 cm) connected to a Magstim 200 magnetic stimulator (Magstim, Whitland, Dyfed, UK). The coil was placed tangentially to the scalp with the handle pointing backwards and 45 deg lateral to the midline. For each subject, the optimal scalp positions to induce the motor response for both the right and the left APB were determined.

The resting motor threshold (rMT) was defined as the minimal stimulator output eliciting MEPs of $>$50 μV in 5 out of 10 consecutive pulses (Rossini *et al.* 1994). Complete muscle relaxation was continuously monitored by visual feedback of the surface EMGs. For each subject, the peak-to-peak amplitudes of the MEPs were measured in each single trial and averaged to evaluate the cortico-spinal excitability.

PBS

The PBS intervention consisted of paired TMS over each hemisphere; the first TMS over the right M1 (the conditioned side) was followed by the second TMS over the left M1 (the targeted side) with an ISI of 15 ms. In total, 180 repetitions of the paired TMS were delivered every 10 s (0.1 Hz) over a period of 30 min. The stimulus intensity was 120% of the rMT for each M1 area. If the PBS intervention was repeated, the experiments were separated by at least 1 day (Fig. 1).

Experimental protocol

We performed several experiments to examine the effects of PBS. The order of the side and the intensities of the TMS were randomized in all experiments. The measurements took ∼10 min. The evaluations were done before, immediately after, 20 min after and 40 min after the PBS in the following experiments (designated as the 'pre', 'post-0', 'post-20' and 'post-40' conditions, respectively; Fig. 1) unless otherwise stated.

Experiment 1: Effects of PBS on motor excitability

The recruitment of the cortico-spinal projection (I–O function) from the left hand M1 was measured in 10 subjects, to investigate the motor cortical excitability. The intensities of the single TMS stimuli were individually adapted according to the rMT to evaluate the I–O function.

Eight MEPs were recorded from the right APB muscle at intensities of 50, 70, 80, 90, 100, 110, 120, 130 and 150% of the rMT, respectively.

Experiment 2: Effects of PBS on rMT and silent period (SP)

In seven subjects, the effects of PBS on the rMT and the duration of the cortical SP at the left M1, which was targeted by the PBS, were further studied. To assess the cortico-spinal excitability, the amplitudes of the MEPs were measured with the fixed intensity of the TMS machine adjusted to produce an MEP of ∼1 mV from both the right and the left APB muscle (stimulus intensity, SI 1 mV) before the PBS.

To investigate the cortical inhibitory system, the SP of the left M1 with a stimulation intensity of 120% of the rMT (before the PBS) was assessed using surface electromyographic recordings of the APB isometrically contracted at 15% of the maximum force. The duration of the SP was defined as the time from the onset of the magnetic stimulation until the return of voluntary electromyographic activity. The force was measured using a force transducer (range, 0–50 lbs; diameter of contact surface area, 2 cm) and was fed back into an oscilloscope. The individual 15% force level was marked directly on the oscilloscope screen in front of the subject.

Experiment 3: Effects of PBS on H-reflex

The H-reflex in the right FCR muscle was measured to test the effects of the PBS at the spinal level in six subjects. To produce an H-reflex in the FCR muscle, the right median nerve was stimulated at the elbow with an electric pulse of 1 ms duration during wrist flexion at 20% of the maximum force. The stimulus intensity was gradually increased from a level below the H-wave threshold to a level at which a stable maximum M-wave was elicited. Both H-waves and M-waves were recorded. The spinal excitability was assessed by the ratio of the maximum H-reflex amplitude (H_{max}) to the maximum M-wave amplitude (M_{max}).

The FCR muscle was selected because reliable H-reflexes cannot be produced in the APB muscle. To confirm that the change in the FCR motor excitability was comparable to that in the APB following the targeted PBS, we also evaluated the MEP of the right FCR in this experiment.

Experiment 4: Effects of PBS on motor behaviour

The effects of the PBS on motor behaviour were examined using the choice reaction time (cRT) task, nine-hole peg test (9HPT; Mathiowetz *et al.* 1985), pinch force and grip

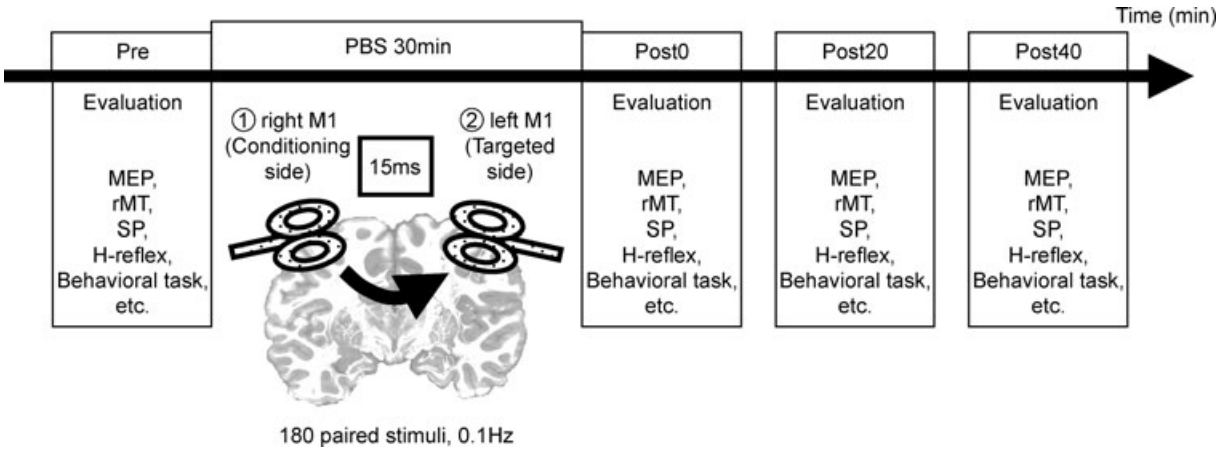

Figure 1. Design of the main experiments
The PBS consisted of 0.1 Hz repetitions of 180 paired stimulations of the TMS at the right M1, followed by the TMS at the left M1, with a delay of 15 ms. We examined motor functional parameters (for example, the amplitudes of MEP, rMT, SP and behavioural tasks in each experiment) before, immediately after, 20 min after and 40 min after the end of PBS (the pre, post-0, post-20 and post-40 conditions, respectively).

power, as well as by measuring the MEPs of the right APB with an SI of 1 mV in 10 subjects. All of the behaviour tasks were assessed for both hands.

In the cRT task, the subjects had to select one of two button-press responses with the right or left thumb, according to the direction of an arrow presented on a monitor set 1 m in front of them. When an arrow was presented, the subjects had to respond as quickly and accurately as possible. The probability of appearance of each of the right and left arrows was set at 50%. Following one or two practice trials, data from 40 trials were collected for each subject at each session. The mean reaction times (RTs) of each hand for a subject were calculated. Incorrect responses and RTs of >500 ms were excluded.

The 9HPT is a standardized quantitative test requiring the accurate rotation and translation with stabilization of the arm and hand. In this test the subjects were presented with a wooden block containing nine empty holes and a small, shallow container holding nine pegs. Upon receiving the start command, the subjects picked up the nine pegs one by one with one hand as quickly as possible, put them into the nine holes and then removed them again one by one as quickly as possible, returning them to the shallow container. The total time taken to complete the task was recorded.

The maximal pinch force using the thumb and index finger of each hand was measured using the same force transducer as that used in Experiment 2. The maximal grip power (kilograms) of each hand was measured. The order of the tasks was randomized both within and across subjects.

In addition, we repeated the experiment with the PBS targeted to the right M1 in seven different subjects, in order to confirm that the behavioural differences were not simply due to the difference in dexterity between the dominant and non-dominant sides in motor learning.

Experiment 5: Site specificity of PBS

PBS of the left M1 (target) and the right parietal area (conditioning site) was performed to test the site specificity of the conditioning stimulus with an ISI of 15 ms in six subjects who had participated in Experiment 1. The conditioning TMS of the right parietal area was applied at position P4 on the electroencephalogram according to the International 10–20 System, which was approximately 6 cm posterior to the right hand M1.

The cortico-spinal excitability was assessed by the mean amplitudes of 20 MEPs recorded from the right APB with SI 1 mV measured before the PBS.

Experiment 6: Differences of the effects of side

To examine the differences of the effects of side, the serial order of the right and left M1 stimulation was reversed during the PBS in six subjects. For the right M1 (the targeted side), 20 MEPs recorded from the left APB with SI 1 mV were also measured. These were compared to the MEPs with SI 1 mV from the right APB before and after normal ordered PBS for the left M1 (the targeted side), which was performed on a different day in the same six subjects.

Experiment 7: Influence of interstimulus interval (ISI) on PBS

PBS was performed with different ISIs (-25, -15, -5, 5, 15 and 25 ms) to test the effect of the timing of the paired stimulation in six subjects. The ISI was defined as the time interval between the TMS of the conditioned side and the targeted side. A minus value for the ISI indicated that the serial order of stimulation during the PBS was reversed; for example, the experimental condition using TMS of the right M1 followed by TMS of the left M1 with a delay of 15 ms was regarded as having an ISI of 15 ms for the MEP of the right APB and -15 ms for that of the left APB.

The mean amplitudes of 20 MEPs for the right and left APB with SI 1 mV were measured. The order of the experiments using different ISIs was randomized across subjects. The effects of the PBS on the left and right M1 were measured separately. To clarify the effects of different ISIs on the PBS, the ratio for MEPs in the post-0 condition compared with the baseline (post-0/pre) condition was measured for each ISI.

Experiment 8: Influence of conditioning intensity

To test whether the conditioning stimulus had the optimum intensity value, PBS was applied with an intensity of 90% of the rMT on the conditioning side in six subjects. The mean amplitudes of 20 MEPs for the right APB with SI 1 mV were measured.

Statistical analysis

The paired *t* test was used to compare the rMT and the test intensities for SI 1 mV between both M1 areas. To assess the effects of PBS, data on the MEP amplitudes for the right and left M1, the intensity for the rMT, the duration of the SP, and the behavioural performance measured in the right and left hand were subjected to repeated-measures ANOVA with Time (pre, post-0, post-20 and post-40) as a within-subject factor. In addition, for Experiment 1, two-way repeated-measures ANOVA was performed using Intensity (50, 70, 80, 90, 100, 110, 120, 130 and 150%) and Time. For Experiment 7, two-way repeated-measures ANOVA was performed using Side (right and left M1) and ISI.

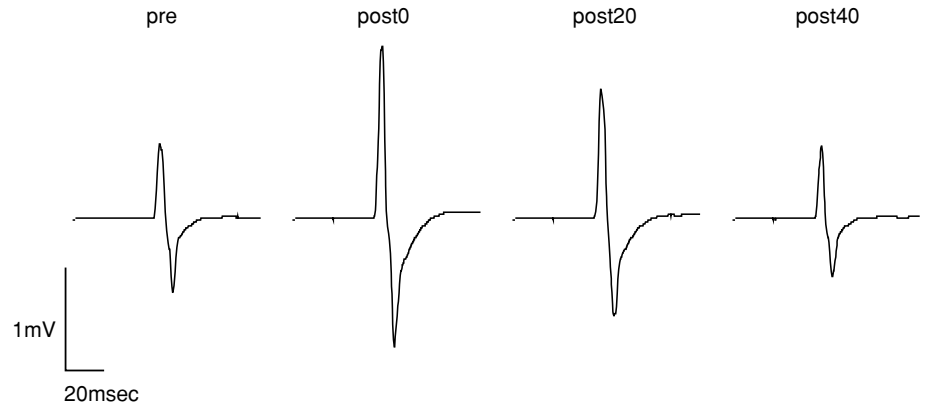

Figure 2. Mean MEP waveforms before and after PBS in one subject
MEPs were recorded from the right APB with a stimulus intensity of 120% of the rMT and were averaged (8 trials for each). MEPs were facilitated immediately after and 20 min after PBS (post-0 and post-20 conditions) compared with the baseline (pre condition), and returned to the baseline 40 min after PBS (post-40 condition).

If necessary, the Greenhouse–Geisser correction was used to adjust for the sphericity, changing the degrees of freedom using a correction coefficient epsilon. The Bonferroni correction for multiple comparisons was used for the *post hoc t* test. Effects were considered significant at $P < 0.05$. All data are given as the mean ± S.E.M.

Results

None of the subjects experienced any side effects from TMS during the experiments.

The rMTs for the right and left APB muscles were 49.5 ± 6.0 and 50.7 ± 7.5% of the maximum stimulator output, respectively ($n = 23$). There was no significant main effect of Side. The test intensities for SI 1 mV for the right and left APB were 62.1 ± 9.6% (Experiments 2–8) and 64.8 ± 9.1% (Experiments 2, 4, 6 and 7), respectively.

Experiment 1: Effects of PBS on motor excitability

Figure 2 illustrates the waveforms of the mean MEPs from one representative subject with an intensity of 120% of the rMT before, immediately after, 20 min after and 40 min after the intervention. The MEPs recorded from the right APB were enhanced immediately after and 20 min after the intervention.

Repeated-measures ANOVA showed significant main effects of Time ($F = 16.63$, $P < 0.001$), and the Time × Strength interaction ($F = 2.0$, $P = 0.029$). The MEP amplitudes as a function of the TMS strength were significantly different in the post-0 and post-20 conditions compared with the pre condition (pre *vs.* post-0, $F = 27.0$, $P < 0.001$; pre *vs.* post-20, $F = 9.3$, $P = 0.003$; Fig. 3). The *post hoc t* test revealed significant increases of the MEP amplitude compared with the pre condition at intensities of 90, 100, 110, 120, 130 and 150% of that of the post-0 condition, and 120% of that of the post-20 condition

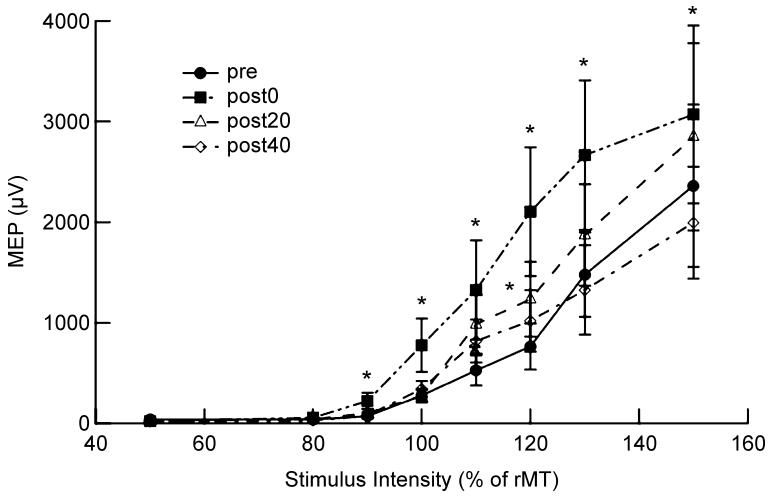

Figure 3. MEP amplitudes as a function of TMS intensity before and after PBS
The mean MEP amplitudes were calculated for the right APB with TMS at intensities of 50, 70, 80, 90, 100, 110, 120, 130 and 150% of the rMT (8 trials for each; 10 subjects). The *post hoc t* test showed an increase of the MEP amplitudes compared with the pre condition at intensities of 90, 100, 110, 120, 130 and 150% of the post-0 condition, and 120% of the post-20 condition (*t* test, $^*P < 0.05$).

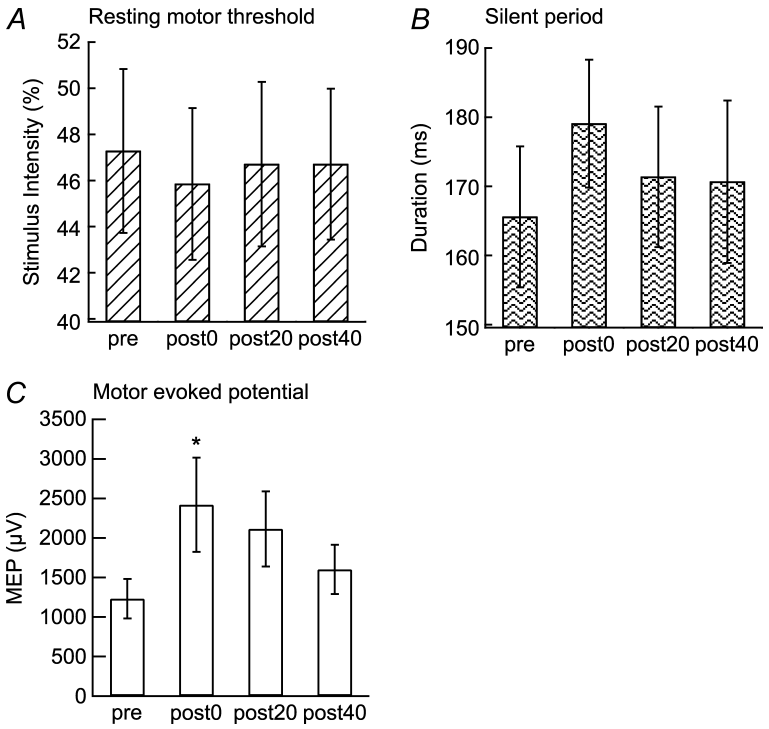

Figure 4. Effects of PBS on rMT and SP
PBS did not have any significant effects on the rMT (*A*) or SP (*B*) of the right APB, but significantly enlarged its MEP amplitudes (*C*) (*t* test, $^*P < 0.05$).

($P = 0.026$, 0.05, 0.024, 0.001, 0.01, 0.047 and 0.032, respectively).

Experiment 2: Effects of PBS on rMT and SP

The rMTs and SPs for the left M1 had a tendency to decrease or increase, respectively, but neither was significantly modulated by the PBS (rMT, $F = 1.36$, $P = 0.29$; SP, $F = 1.79$, $P = 0.19$; Fig. 4*A* and *B*). The MEPs for the right APB muscle were significantly increased by the PBS ($F = 4.96$, $P = 0.01$; *post hoc t* test, pre *vs.* post-0, $P = 0.042$; Fig. 4*C*). The MEPs for the left APB muscle were not significantly modulated by the PBS ($F = 0.78$, $P = 0.5$).

Experiment 3: Effects of PBS on H-reflex

The mean H-latency was 15.78 ± 0.79 ms. There was no significant change in the H_{max}/M_{max} ratio (Fig. 5*A*). The amplitudes of the MEPs of the right FCR muscles, as well as the right APB, were significantly enhanced immediately after the PBS (FCR, $F = 4.3$, $P = 0.022$; pre *vs.* post-0, $P = 0.001$; APB, $F = 3.4$, $P = 0.046$; *post hoc t* test, pre *vs.* post-0, $P = 0.005$; Fig. 5*B*).

Experiment 4: Effects of PBS on motor behaviour

For the PBS targeting the left M1, a significant decrease in the completion time in the 9HPT was found in the right hand in the post-0, post-20 and post-40 conditions ($F = 10.0$, $P = 0.0004$; *post hoc t* test, pre *vs.* post-0, $P = 0.005$, pre *vs.* post-20, $P = 0.003$, and pre *vs.* post-40, $P < 0.0003$; Fig. 6*A*) and in the post-40 condition in the left hand ($F = 5.3$, $P = 0.005$; *post hoc t* test, pre *vs.* post-40, $P = 0.0045$; Fig. 6*B*).

For the pre, post-0, post-20 and post-40 conditions, respectively, the cRT (right hand, 353.2 ± 8.6, 338.9 ± 8.2,

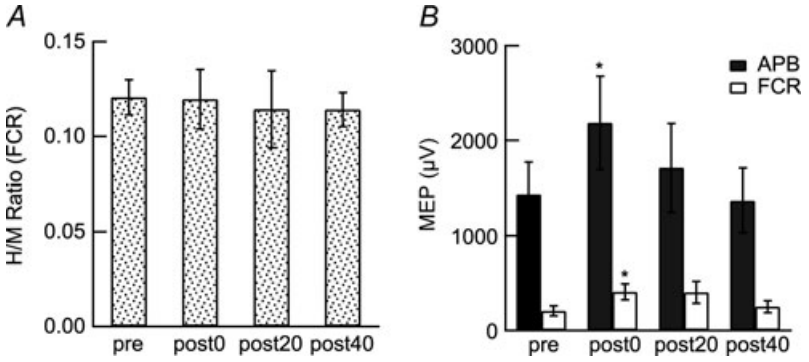

Figure 5. Effects of PBS on H-reflex and FCR muscles
A, the H-reflex in the right FCR muscles was measured by stimulating the right median nerve at the elbow during wrist flexion at 20% of the maximum force. The H_{max}/M_{max} ratio was not significantly changed after PBS. *B*, the MEP amplitudes for the right FCR, as well as the APB, were significantly enhanced immediately after the PBS (*t* test, $^*P < 0.05$).

342.7 ± 6.0 and 338.3 ± 10.0 ms; left hand, 349.8 ± 9.0, 343.7 ± 8.6, 340.7 ± 4.1 and 340.1 ± 7.6 ms), pinch force (right hand, 12.9 ± 0.6, 14.2 ± 0.8, 13.7 ± 0.7 and 13.9 ± 1.0 lbs; left hand, 12.4 ± 0.8, 11.9 ± 0.8, 12.6 ± 0.6 and 12.9 ± 0.6 lbs) and grip power (right hand, 36.0 ± 1.6, 37.0 ± 1.5, 37.1 ± 1.2 and 36.4 ± 1.5 kg; left hand, 34.4 ± 1.5, 34.6 ± 1.5, 35.7 ± 1.4 and 34.8 ± 1.5 kg) were not significantly modified by the PBS.

For PBS targeting the right M1, a significant decrease of the completion time in the 9HPT was found in the left hand in the post-0, post-20 and post-40 conditions ($F = 14.9$, $P < 0.0001$; *post hoc t* test, pre *vs.* post-0, $P = 0.01$, pre *vs.* post-20, $P < 0.001$, and pre *vs.* post-40, $P < 0.001$; Fig. 6*C*), whereas no significant change was observed in the right hand ($F = 5.0$, $P = 0.03$; *post hoc t* test, pre *vs.* post-0, $P > 0.05$, pre *vs.* post-20, $P > 0.05$, and pre *vs.* post-40, $P > 0.05$; Fig. 6*D*).

For the pre, post-0, post-20 and post-40 conditions, respectively, the cRT of the left hand was also significantly modified by PBS targeting the right M1 (393.9 ± 17.4, 370.0 ± 18.7, 370.1 ± 14.7 and 377.3 ± 20.0 ms; $F = 4.2$, $P = 0.02$; *post hoc t* test, pre *vs.* post-0, $P = 0.02$, and pre *vs.* post-20, $P = 0.02$), while that of the right hand was not (380.4 ± 14.4, 376.7 ± 16.1, 369.7 ± 16.4, 364.7 ± 18.2 ms; $F = 1.3$, $P > 0.05$).

For the pre, post-0, post-20 and post-40 conditions, respectively, the pinch force (left hand, 11.9 ± 1.1, 11.7 ± 0.8, 12.8 ± 1.6 and 12.3 ± 1.3 pounds; right hand, 13.7 ± 1.3, 12.4 ± 0.8, 11.9 ± 1.0 and 12.8 ± 1.1 pounds) and grip power (left hand, 40.5 ± 3.2, 38.6 ± 2.6, 38.9 ± 2.7 and 35.4 ± 2.0 kg; right hand, 42.6 ± 2.8, 41.7 ± 2.7, 41.5 ± 3.4 and 41.2 ± 1.3 kg) were not significantly modified.

Experiment 5: Site specificity of PBS

When the conditioning TMS of the PBS was applied to the right parietal area, the MEPs for the right APB were not significantly modulated (1042.2 ± 310.6, 653.1 ± 245.2, 792.4 ± 203.6 and 974.0 ± 263.9 μV for the pre, post-0, post-20 and post-40 conditions, $F = 1.3$, $P = 0.3$).

Experiment 6: Differences of side

The MEP change in the right APB induced by PBS targeted to the left M1 was compared with that in the left APB induced by PBS targeted to the right M1. Time had a significant effect ($F = 4.57$, $P = 0.023$), but the Time × Side interaction did not ($F = 2.76$, $P = 0.08$; Fig. 7).

Experiment 7: Influence of ISI on PBS

PBS targeting the left or right M1 with an ISI of 15 ms produced a significant increase of MEP amplitudes only in the right APB ($F = 4.0$, $P = 0.029$; *post hoc t* test, pre *vs.*

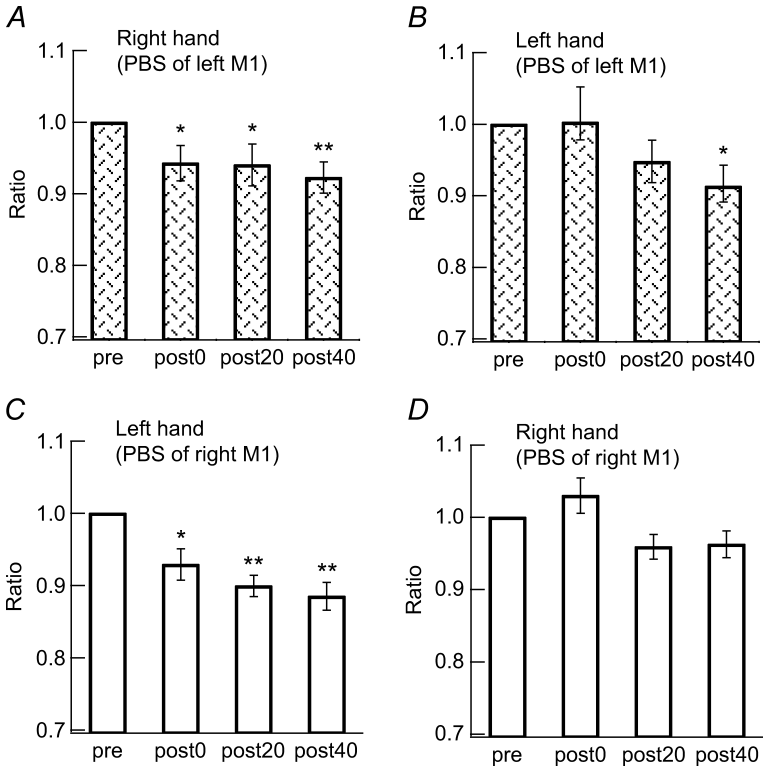

Figure 6. Effects of PBS on time taken to complete 9HPT
The times required to complete the 9HPT by the right hand (*A*) and the left hand (*B*) were measured. A significant enhancement was found in the right hand immediately after PBS (post-0 condition), which continued for over 40 min (post-40 condition), whereas in the left hand it was seen only at 40 min post-PBS (post-40 condition) (*t* test, $^*P < 0.05$).

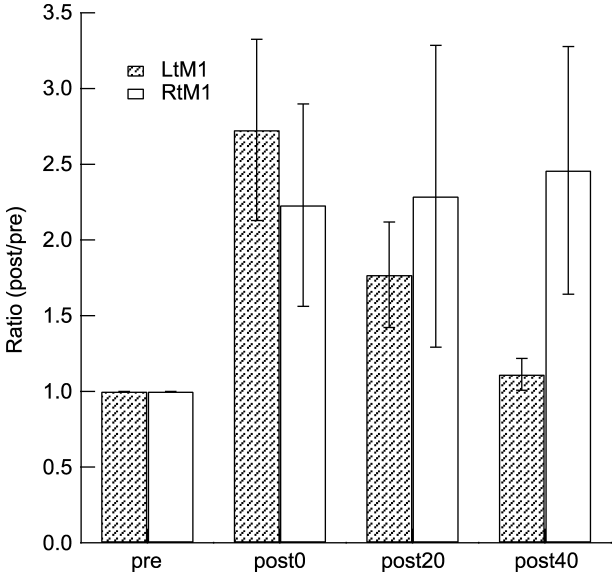

Figure 7. Effects of PBS targeting the left and right M1
The effects of PBS targeting the left M1 measured by the MEP amplitudes of the right APB, and the right M1 measured by the MEP amplitudes of the left APB, are shown. There was no significant interaction between Side (right and left) and Time (pre, post-0, post-20 and post-40). For presentation purposes, the ratio of MEPs compared to the pre condition is shown here.

post 0, $P = 0.009$) and the left APB ($F = 3.9$, $P = 0.031$; *post hoc t* test, pre *vs.* post-0, $P = 0.021$). PBS with different ISIs (−25, −15, −5, 5 and 25 ms) did not induce any significant change in the MEP amplitudes.

Two-way repeated-measures ANOVA of the MEP ratio (post-0/pre) demonstrated a significant effect of the ISI ($F = 5.6$, $P = 0.002$; Fig. 8). The interaction of Side × ISI was not significant ($F = 1.1$, $P = 0.4$).

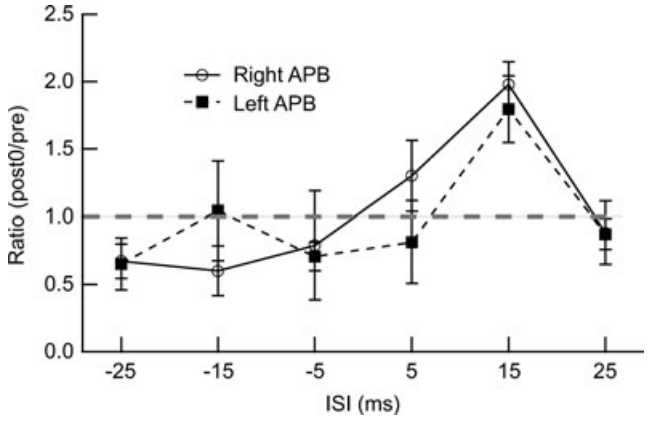

Figure 8. Influence of ISI on effects of PBS
The ratio of the MEP amplitudes of the right or left APB muscles (post-0/pre) is presented as a function of the ISI measured from the time of conditioning stimulation to that of target M1 stimulation. The largest MEP ratio was observed at an ISI of 15 ms.

Experiment 8: Influence of conditioning intensity

When the conditioning stimulus intensity was fixed at 90% of the rMT and the target intensity was 120% of the rMT during the PBS, the MEPs for the right APB were not significantly modulated (1526.2 ± 38.1, 1459.9 ± 496.2, 1416.3 ± 666.7 and 1473.3 ± 358.6 μV for the pre, post-0, post-20 and post-40 conditions, respectively).

Discussion

The present results showed that paired low-frequency TMS over bihemispheric motor areas with a specific time interval (PBS) produced transiently sustained increments of motor cortical excitability and enhanced motor performances on the targeted side. The effects of the PBS were temporary and reversible; they developed immediately, continued for a period (20–40 min) and then returned to the baseline. PBS seemed to show timing specificity between two different inputs. Thus, it is likely that PBS combining TMS and interhemispheric projection induces associative LTP-like changes in the human motor system.

Extending a previous study by Rizzo *et al.* (2009), we found that the bihemispheric stimulation of homologous areas (that is, M1–M1 but not the parietal area–M1) was necessary to produce plasticity. The behavioural consequences of PBS were found to include an improvement in the performance of the 9HPT as well as the cRT task. The H-reflex experiment demonstrated that PBS did not modify the spinal excitability as measured by the H_{max}/M_{max} ratio. Therefore, it is likely that the increase in motor excitability after PBS was produced at the supraspinal level. In addition, we found that the effects of PBS were timing dependent. The increase of excitability was dependent on the specific time interval of the activation of the target M1 through two afferent pathways.

Regarding the behavioural consequences, PBS produced significant changes in the complex fine motor control of digits measured by the 9HPT for the target hand, irrespective of the side of the intervention. The improvement was observed immediately after PBS and continued over 40 min in the target hand. It was not clear why there was enhanced motor performance without a significant increase of the MEP amplitude in the target hand at 40 min post-PBS. One hypothesis is that the PBS-induced plasticity might have enhanced the learning rate or the consolidation of motor learning induced by repeating the 9HPT during the experiment. If this was the case, the improved motor performance might have lasted longer than the change in motor excitability measured by the MEP amplitudes. Repetitive TMS was reported to interfere with the consolidation of motor learning (Muellbacher *et al.* 2000). It was likely that motor learning contributed to the 9HPT performance change, especially

during the later part of the experiment, because similar enhancement occurred in the left (non-target) hand at 40 min post-PBS targeting the left M1.

Alternatively, the neural circuits responsible for the 9HPT performance might only partially overlap with the cortico-spinal neurons associated with the MEP generation. If so, the MEP amplitudes and motor performance of the 9HPT might reflect the different aspects of human motor function, and could behave differently following PBS.

For the cRT task, we found that PBS targeting the right M1 produced a shortening of the RT, which was consistent with the study by Rizzo *et al.* (2009). The change of the RT in the right hand for PBS targeting the left M1 showed a similar tendency, but failed to reach the level of significance. As dexterity should be superior in the side of the dominant hand, it is possible that the behavioural effects of PBS might be more clearly demonstrated for the left hand.

In primate studies, lesions in the primary sensorimotor or motor area have produced specific failures of fine finger movements such as the precision grip (Passingham *et al.* 1983; Liu & Rouiller, 1999; Murata *et al.* 2008), suggesting that the M1 hand area is essential for exerting skilled fine movements. From this viewpoint, it is reasonable to assume that the 9HPT is a more sensitive measure of M1 function than the cRT, power grip or pinch force used in the present study.

In human studies, an enhancement in motor performance has been also reported along with the increased cortico-spinal excitability produced by TMS in healthy adults (Pascual-Leone *et al.* 1998; Butefisch *et al.* 2004; Kim *et al.* 2004). These findings are also consistent with the observed effects of PBS in our motor behaviour tests. Previous studies in stroke patients showed that high-frequency repetitive TMS could enhance the cortico-spinal excitability of the affected hemisphere and improve the activities of daily life in the acute stage (Khedr *et al.* 2005), as well as the accuracy and speed of the affected hand movement in the chronic stage (Kim *et al.* 2006). The effects of PBS on cortico-spinal excitability and motor behaviour, and especially on hand dexterity, suggest that it might be useful in stroke rehabilitation.

Although neuronal plasticity has been recognized to occur at multiple levels of the central nervous system, the PBS-induced changes in the present experiment were likely to have occurred at the supraspinal level, because the H_{max}/M_{max} ratio did not change significantly, suggesting that the excitability at the spinal level was insensitive to PBS. Testing the spinal excitability is particularly important for understanding the physiological mechanism of PBS, because ipsilateral projection to the spinal motor neuron has been reported in anatomical and physiological studies (Shahani & Young, 1971; Roby-Brami & Bussel, 1987; Delwaide & Pepin, 1991; Nathan *et al.* 1996). It is possible that paired bihemispheric cortical stimulation can produce paired afferent inputs to the spinal motor circuits, which might cause plasticity only at the spinal motoneurons that receive descending inputs but are not involved in the generation of H-reflex.

A recent study by Meunier *et al.* (2007) showed that conventional PAS produced the changes in spinal excitability measured by H-reflex recruitment curves. Thus, it is also possible that MEPs and H-reflexes might not necessarily reflect the same motor neuronal pools. However, Nielsen *et al.* (1993) and Petersen *et al.* (2003) reported on the modulation of the H-reflex induced by TMS, suggesting that the TMS recruits the same motor neuronal pool that receives the afferent input from the H-reflex. As we could not find any change in the H-reflex following PBS, it was likely that the contribution of the spinal plasticity might have been smaller for our PBS protocol than for the conventional PAS protocol combining peripheral nerve stimulation and TMS.

Di Lazzaro *et al.* (2009) reported on the enhancement of the amplitudes of the descending volleys evoked by TMS using cervical epidural recording following the conventional PAS protocol, which provided direct evidence of the plasticity at the cortical level. To understand fully the cortical and spinal contribution to the PBS-induced plasticity, further studies in these comparatively rare patients or animal models will be necessary.

Moreover, the MEP amplitudes as a function of the stimulus intensity (I–O function), which are thought to represent the motor cortical excitability (Ridding & Rothwell, 1997), were affected by PBS for up to 20 min post-PBS. The I–O function reflects not only the size of the population of firing neurons activated by the suprathreshold stimuli, but also the excitability of the neurons produced by the subthreshold stimuli. A change of the I–O function means a change of the excitability of the motor representation, with increasing or decreasing numbers of firing neurons seen at varying stimulus intensities (Ridding & Rothwell, 1997). The change of I–O function in the present study also supported the hypothesis that PBS produced motor cortical plasticity.

The rMT, which reflected the neuronal membrane excitability level in the motor cortex (Mavroudakis *et al.* 1994; Ziemann *et al.* 1996; Hallett, 2000), showed a tendency to decrease. By contrast, the SP, the later part of which represented the cortical inhibitory system (Inghilleri *et al.* 1993; Chen *et al.* 1999), showed a tendency to increase. However, both failed to reach the level of significance.

PBS seems to share various features with 'associative LTP', which has been previously reported in animal studies. Low-frequency presynaptic stimulation coupled with concurrent postsynaptic depolarization was reported to induce LTP in hippocampal slices from rats (Kelso

& Brown, 1986; Robinson, 1986; Sastry *et al.* 1986). In cat studies, pairing the stimulation of various cortical afferents including the callosal system with depolarization (Baranyi & Szente, 1987) or stimulation-induced firing of the postsynaptic cells in the motor cortex (Baranyi & Feher, 1981) produced associative LTP, which required the coupled stimulation of different afferent pathways.

In our current PBS protocol, the plastic changes were induced only when conditioning TMS was applied 15 ms prior to TMS of the target M1, suggesting timing-dependent plasticity according to the Hebbian rule (Hebb, 1949). Rizzo *et al.* (2009) found LTP-like effects using a similar protocol with an ISI of 8 ms. We found an absence of LTP-like effects at ISIs of 5 and 25 ms, and so the critical time window for LTP might be within this range. Although conventional PAS studies using TMS and peripheral nerve stimulation showed bidirectional plasticity depending on the ISI of two stimuli (Wolters *et al.* 2003, 2005), we could not find an associative LTD-like effect in the present experimental setting. A previous study also failed to produce an associative LTD-like effect using an ISI of 1 ms (Rizzo *et al.* 2009). However, there was a non-significant tendency towards LTD-like effects when the conditioning stimulus was applied 5–25 ms after the test stimulus. It was possible that the associative LTD-like effect might have been difficult to observe due to the narrower time window within which the ISI could induce depressive effects.

Although the time interval between the presynaptic input and the postsynaptic firing is critical for the modification of synaptic strength, the stimulus parameters that can effectively produce the associative plasticity might vary depending on the types of synapse studied. Some previous animal experiments reported on the importance of the specific time interval (Dan & Poo, 2004, 2006; Caporale & Dan, 2008). LTP in excitatory postsynaptic potentials (EPSPs) was produced when postsynaptic action potentials (APs) were preceded by 10 ms, while LTD in the EPSPs was produced when the APs were delayed by 10 ms, and critical time window was −100 ms to about ∼100 ms for the AP–EPSP interaction in cortical synapses of rats (Markram *et al.* 1997). In the cerebellum-like structure of fish, EPSPs were enhanced after postsynaptic spikes preceded EPSPs onset by 8∼90 ms, while EPSPs were depressed after the postsynaptic spikes followed EPSPs onset within 60 ms, as though according to an anti-hebbian rule (Bell *et al.* 1997). LTP was induced when single presynaptic APs preceded the postsynaptic APs by 15 ms, while LTD was induced when single presynaptic APs were synchronously paired or asynchronously delayed with postsynaptic APs by 25∼200 ms in CA3 pyramidal cells of rats hippocampus (Debanne *et al.* 1998). LTD was produced when the presynaptic excitatory input arrived between 10 ms before and 25 ms after the postsynaptic firing in layer 4 of the somatosensory cortex of rats (Egger *et al.* 1999). If the inhibitory presynaptic input arrived 410–510 ms after the onset of the postsynaptic firing, LTP occurred in presynaptic inhibition, whereas LTD occurred if the presynaptic input arrived up to 250 ms after the onset of the postsynaptic firing in the neocortical pyramidal cells of rats (Holmgren & Zilberter, 2001). In cell cultures of hippocampal glutamategic neurons, repetitive post-synaptic spiking within a time window of 20 ms after presynaptic activation resulted in long-term potentiation (LTP), whereas postsynaptic spiking within a window of 20 ms before the repetitive presynaptic activation led to long-term depression (LTD) (Bi & Poo, 1998).

The induction of LTP in the mature M1, especially in the horizontal pathways, might require the additional modulation of the vertical (thalamocortical and cortico-cortical) inputs (Hess *et al.* 1996; Hess & Donoghue, 1996). As for the associative LTP-like effects observed in the present study and by Rizzo *et al.* (2009), TMS applied to the target M1 is known predominantly to activate the cortico-spinal neurons in the M1 transsynaptically (Di Lazzaro *et al.* 2004). Altering the target M1 activity induced by the direct and indirect cortico-cortical projections connecting both M1 areas (Gerloff *et al.* 1998) might work as an associative factor to produce LTP-like modulation of the synaptic efficacy in the target M1.

Another possible mechanism for the LTP-like effect of PBS is persistent non-synaptic neuronal change in intrinsic excitability due to the altered membrane conductance, which has been reported in brain tissue from several species during the enhancement of learning and memory (Sah & Bekkers, 1996; Moyer *et al.* 2000; Saar *et al.* 2001, 2002; Stackman *et al.* 2002; Barkai, 2005). Further studies will be necessary to determine whether the PBS-induced plasticity is the same phenomenon as the associative plasticity at the cellular level. Nonetheless, the present results, as well as those of Rizzo *et al.* (2009), confirmed that the motor threshold of the target M1 was not significantly affected by PBS. The MEP threshold is a general measure of cortico-spinal excitability, and is closely linked to the neuronal and inter-neuronal membrane excitability, which can be altered by sodium channel blockers (Ziemann *et al.* 1996).

Rizzo *et al.* (2009) reported on the asymmetry of the cortico-spinal excitability change using a similar inter-vention protocol. PBS targeting the right, but not the left, M1 produced LTP-like effects. However, in the present study, there was no significant difference in the effect of PBS. Moreover, the LTP-like effect as a function of the ISI was also similar for the right and left M1. One possible reason for this divergence might have been the difference in the PBS protocol. The frequency of paired stimulation was higher (0.1 *vs.* 0.05 Hz) and the ISI was longer (15 *vs.* 8 ms) in our current study. As various inhibitory and excitatory neural circuits are involved in the generation of inter-hemispheric interaction (Daskalakis *et al.* 2002; Chen *et al.*

2003; Lee *et al.* 2007), PBS using different ISIs might preferentially activate different neuronal populations within and between the M1 areas. In this regard, the neural basis of the LTP-like effects observed in the present study and by Rizzo *et al.* (2009) might not be exactly the same. Further research evaluating the afferent inhibition or intracortical inhibition/facilitation of short or long latencies will be useful to clarify this point. However, it is reasonable to assume that the asymmetric plasticity of two hemispheres might reflect the quantitative, but not qualitative, differences, and that a sufficiently powerful protocol could produce LTP-like effects in the right and left M1.

As we were able to induce LTP-like effects immediately after the intervention in the left and right M1, it is likely that PBS with an ISI of 15 ms is better at producing plasticity than that with an ISI of 8 ms. The ISI should be a relevant parameter that determines the plasticity, because the effectiveness of associative LTP is often dependent on the spike timing (Hess *et al.* 1996; Hess & Donoghue, 1996; Bell *et al.* 1997; Bi & Poo, 1998; Holmgren & Zilberter, 2001). In studies of interhemispheric inhibition using TMS (Ferbert *et al.* 1992), a double shock with an ISI of 6–20 ms had an inhibitory effect mainly in the M1, possibly through the transcallosal pathway. In addition, the conduction time estimated from the interhemispheric inhibition of the MEP was similar to those investigated by the human transcallosal electroencephalographic response (Cracco *et al.* 1989) or in patients with cortical myoclonus (Shibasaki *et al.* 1978; Wilkins *et al.* 1984; Brown *et al.* 1991; Hanajima *et al.* 2001).

In a previous study, Rizzo *et al.* (2009) showed significant attenuation of the interhemispheric inhibition (IHI) with ISIs of 10 and 40 ms induced by PBS with an ISI of 8 ms, suggesting that the facilitation of MEP amplitudes was due to the reduction of the IHI. Thus, modulation of the IHI is one possible mechanism underlying the PBS-induced plasticity observed in the present study. However, it is also possible that the excitability of a specific subset of intracortical interneurons in the targeted M1 was altered. The interhemispheric projection and various intracortical interneurons are known to interact with each other (Daskalakis *et al.* 2002; Chen *et al.* 2003; Lee *et al.* 2007).

The IHI with an ISI of ∼10 ms was probably due to excitatory inputs from transcallosal fibres to the contralateral cortical inhibitory interneurons, as the transcallosal and descending cortico-spinal tract seem to have different origins in some animal studies (Chang, 1953; Jacobson & Marcus, 1970; Jacobson & Trojanowski, 1974; Catsman-Berrevoets *et al.* 1980). Moreover, although the cortico-spinal neurons are known to originate from layer V of the M1, the transcallosal neurons of the M1 are localized in layers II–VI (Jacobson & Trojanowski, 1974; Catsman-Berrevoets *et al.* 1980). So far, direct cortico-cortical interhemispheric projections to large pyramidal cells in layer V have not been demonstrated in animal studies (Chang, 1953; Jacobson & Marcus 1970).

It is not clear why the associative LTP-like effects in humans, such as PAS or PBS, are induced by the afferent stimulation, which by itself has inhibitory effects on the M1. Median nerve stimulation used in PAS is known to produce afferent inhibition of the MEP at an ISI of ∼20 ms (Inghilleri *et al.* 1990; Tokimura *et al.* 2000). In the case of PBS, contralateral M1 stimulation at an ISI of ∼10 ms can cause interhemispheric inhibition (Ferbert *et al.* 1992; Ni *et al.* 2008). It is possible that the afferent stimulation might affect the interneuron network in the M1 through multiple synaptic pathways, which might partly involve the excitatory system as well as the inhibitory one. Indeed, it should be noted that the exact timing of the paired stimulation of the conventional PAS (Stefan *et al.* 2000) or PBS in the present study was longer than the ISI at which the inhibition of the MEP was maximal. Afferent inhibition of the MEP amplitude was reduced when the ISI between the median nerve stimulation and TMS was 25 ms (Kotb *et al.* 2005). Recently, Ni *et al.* (2008) reported that the IHI showed inhibitory peaks at ISIs of 10 and 40 ms, with a trough or non-significant inhibition at ∼16 ms.

In non-human primates, the transcallosal connections between motor representations of hand area are known to be sparse (Jenny 1979; Pappas & Strick, 1981; Gould *et al.* 1986). However, the stimulation of the corpus callosum in the monkey activated the precentral neurons related to finger and wrist movement orthodromically (Matsunami & Hamada, 1984), suggesting that the connections function in the hand M1. Behaviourally, the human corpus callosum might be important for achieving bimanual coupled movements. Patients with a callosotomy or with an acquired callosal lesion exhibited specific impairments in synchronous bilateral hand movement (Serrien *et al.* 2001; Kennerley *et al.* 2002; Seitz *et al.* 2004). However, their unilateral hand movement, or bilateral but different movement in each hand, was preserved as normal. Moreover, Rizzo *et al.* (2009) demonstrated that the effects of PBS were mainly produced through the transcallosal tract, because a patient with callosal agenesis showed no significant changes of cortico-spinal excitability after the intervention. Therefore, it is likely that the effects of PBS are mediated through the transcallosal tract.

A previous PAS study using median nerve stimulation and TMS reported topographical specificity of the LTP-like effects within the M1, which might have been due to the tight connection of the homologous somatosensory and motor areas (Stefan *et al.* 2000). However, we found that the MEP amplitudes of the FCR were enhanced when the positioning of the coils was optimized for the APB representation area of the hand M1, suggesting

that the effects of PBS were not strictly muscle specific. As cortico-spinal neurons have projections to several nearby muscles (Humphrey *et al.* 1991), the topographic organization of the motor map might be less strict than that of the somatosensory map.

With regard to the inter-regional site specificity of the LTP-like effects, when the conditioned stimuli were applied over the contralateral parietal area that was not directly connected with the targeted M1, no significant LTP-like effects were produced in the present study. This finding strongly suggests that the stimulation of homotopic areas in both hemispheres is necessary for the induction of changes by PBS. However, if PAS is a general way in which to generate associative LTP-like effects in the human brain, it is still possible that the paired stimulation of the parietal area and the M1 at an appropriate ISI might modulate the cortico-spinal excitability.

A threshold intensity of PBS appeared to be required for conditioning TMS in order to induce the LTP-like effects. An intensity of 90% of the rMT is thought to represent a subthreshold level for producing interhemispheric effects in paired TMS (Ferbert *et al.* 1992). It was understandable that PBS using the subthreshold conditioning TMS, which had no modulation effects on the other M1 excitability measured by TMS, failed to produce LTP-like effects. It might be necessary to activate the cortical circuits sufficiently strongly to transmit signals to the targeted area through interhemispheric pathways.

In conclusion, we showed that human LTP-like plasticity at the M1 area could be induced by PBS, suggesting that repeated pairings of stimuli applied to the brain could be an effective general tool for producing plasticity. The present PBS protocol with an ISI of 15 ms might have been more powerful than that used in a previous study by Rizzo *et al.* (2009), because we successfully induced plasticity in both the left and the right M1 to a similar extent. In behavioural and physiological studies in both animals and humans (Rioult-Pedotti *et al.* 2000), the LTP/LTD is likely to be a physiological mechanism underlying learning and memory. Thus, the associative LTP-like changes induced by PBS prove the importance of associative plasticity in human motor control, and have the potential to influence our motor behaviour by modulating the function of the M1. In particular, this methodology might be clinically applied for the rehabilitation of hemiparetic patients (Hummel & Cohen, 2006) or the treatment of movement disorders (Quartarone *et al.* 2003, 2008).

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Author contributions

S.K. contributed to the conception and design of the study, performed the analysis and interpretation of the data, drafted the article and approved the final version. T.M., M.N., Y.U., H.F. and K.D. contributed to the analysis and interpretation of the data, critically reviewed the intellectual content and approved the final version.

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