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Intracortical inhibition of the motor cortex in Segawa disease (DYT5)

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Abstract—*Background:* Segawa disease (autosomal dominant guanosine triphosphate cyclohydrolase I [GTP-I] deficiency, DYT5) is a hereditary dopa-responsive generalized dystonia. *Objective:* To investigate the pathophysiologic mechanisms for dystonia in Segawa disease, we studied intracortical inhibition of the primary motor cortex in patients with Segawa disease. *Methods:* We studied 9 patients with Segawa disease (8 genetically confirmed patients and 1 with abnormally low GTP-I activity) and 12 age-matched normal control subjects. We studied the active motor threshold (AMT) using single pulse transcranial magnetic stimulation (TMS) and the short-interval intracortical inhibition (SICI) of the motor cortex using the previously reported paired pulse TMS method. Responses were recorded from the first dorsal interosseous (FDI) and tibialis anterior (TA) muscles. *Results:* The AMT was not significantly different between the patients and normal subjects. For both studied muscles, in Segawa disease, normal amount of SICI was evoked at interstimulus intervals (ISIs) of 1 to 4 msec even though they had dystonia in those muscles. *Conclusion:* Normal SICI of the motor cortex in Segawa disease stands in remarkable contrast to the previously reported reduction of SICI in focal dystonia. This suggests that the gamma-aminobutyric acid A system of the motor cortex is intact in Segawa disease. The pathophysiologic mechanisms for dystonia must be partly different between Segawa disease and focal dystonia.

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Segawa disease (autosomal dominant guanosine triphosphate cyclohydrolase I [GTP-I] deficiency, DYT5) is a hereditary dopa-responsive generalized dystonia, typically with onset in childhood, and characterized by marked diurnal fluctuation and sustained dramatic responses to relatively low dose levodopa.[1-4] The biochemical basis for the disease is the partial deficiency of tetrahydrobiopterin (BH4) usually due to heterozygous mutation for GCH-I gene located at 14q22-q22.2.[5-8] However, the generator mechanism for dystonia in Segawa disease is unclear.

In some dystonia, the paired pulse transcranial magnetic stimulation (TMS) has demonstrated reduced cortical inhibition. In this method, a preceding subthreshold conditioning stimulus suppresses the motor evoked potentials (MEPs) to a following suprathreshold test stimulus at interstimulus intervals (ISIs) of 1 to 5 msec in normal subjects (short interval intracortical inhibition: SICI). SICI is considered to occur at the primary motor cortex (M1) mediated by its GABAa inhibitory interneurons.[9-11] SICI of the hand motor area was reduced in patients with focal hand dystonia[12-14] and that of the neck motor area in torticollis.[15] Decreased SICI may represent the secondary disinhibition of M1 induced by the primary basal ganglia dysfunction. The disinhibition of M1 is considered to play an important role in the pathogenesis of focal dystonia.

Based on these backgrounds we studied SICI in patients with Segawa disease[9] to investigate whether similar disinhibition occurs in M1 in Segawa disease. We show that the mechanism for dystonia of Segawa disease could be different from that for focal dystonia.

Methods. *Subjects.* We studied 9 patients with Segawa disease (26 to 59 years old, 3 men and 6 women) and 12 normal volunteers (28 to 52 years old, 11 men and 1 woman) (table). Eight patients were confirmed to have an abnormal GCH-I gene, and the other patient (Patient 1) was diagnosed based on clinical symptoms and abnormally reduced GTP-I activity (9.0 pmol/hour/mg protein; normal limit >20 pmol/hour/mg protein).[5] Moreover, the father of this patient (he was not included in this study) also had dopa-responsive generalized dystonia. We could not perform his biochemical analyses because he died some years ago. Six patients (Patients 1 through 6) showed typical symptoms of Segawa disease, i.e., starting with talipes equinovarus in one extremity in childhood and dystonia expanding to all extremities asymmetrically with aggravation of hypertonus. They also showed clumsy pronation/supination movements of upper extremities. Three adult onset patients (Patient 7, 8, and 9) developed some atypical symptoms. The daughters of Patient 7 had typical symptoms of Segawa disease (they did not participate in this experiment because of their ages). Patient 7 showed talipes equinovarus in both legs and upper limb dystonia with stereotypic coarse twist movements at rest. Patient 8 (a younger brother of Patient 3) developed focal hand dystonia. Patient 9 was the father of Patient 2. He had

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Table Subjects

Patient/age*/ sex/onset age*	GCH-I gene abnormality	Symptoms	Levodopa withdrawal†	BFM scale
1/26/F/1	–	L > R talipes equinovarus, L hand dystonia	30	N2
2/33/F/7	+	L > R leg dystonia, L > R hand slowness	18	8
3/40/F/8	+	R > L hand and leg dystonia, induced rigidity torticollis	19	17
4/38/F/9	+	L hand, L > R leg dystonia, scoliosis	24	11
5/44/M/11	+	L > R talipes equinovarus, L hand dystonia	27	0
6/37/F/13	+	L > R leg dystonia, scoliolis	20	10
7/43/F/29	+	L > R talipes equinovarus, L hand dystonia, dyskinesia	32	32
8/39/M/30	+	Writer's cramp	Fresh	7
9/59/M/58	+	Parkinsonism	Fresh	15

* Years.
† Hours.

several parkinsonian features such as masked face, cogwheel rigidity, tremor in the left arm, and bradykinesia. Patients 8 and 9 were studied before the commencement of levodopa therapy. In the other patients, remarkable improvement was obtained with levodopa treatment. In this study, we pooled the data from the fresh patients and those already treated with levodopa because the amounts of SICI were not different between the two groups of patients (see figures 3 and 4). The patients abstained from taking medications for at least 18 to 32 hours prior to the experiments. At the time of examination, the patients showed mild to moderate dystonia in the extremities.

The experiments were performed according to the Declaration of Helsinki and the procedures used were approved by the Ethics Committee of the University of Tokyo. No side effects were noted in any of the individuals.

Paired pulse magnetic stimulation. The SICI of the motor cortex was studied with the technique described in previous reports.[9,13] Surface EMG was recorded from the FDI and TA muscle with Ag-AgCl surface cup electrodes of 9 mm diameter. The active electrode was placed over the muscle belly and the reference electrode over the metacarpophalangeal joint of the index finger or the tendon of TA. Responses were amplified with a Biotop (GE Marquette Medical Systems Japan) through filters set at 100 Hz and 3 kHz, then recorded by a computer (Signal Processor DP-1200, GE Marquette Medical Systems Japan) on which a randomized conditional averaging was performed. In normal subjects, we studied SICI of FDIs on both sides to evaluate its side difference, because it has been reported that the amount of SICI is different between the dominant and nondominant FDIs.[16] We studied the right TA because no side difference was reported for TA. In the patients, we studied the more affected side after cessation of levodopa (i.e., three dominant and five nondominant sides for FDI, and two dominant and seven nondominant sides for TA).

For magnetic stimulation, we used Magstim 200 magnetic stimulators (The Magstim Company, UK) with a circular coil (11.5 cm outer diameter) placed over the vertex. The coil current was adjusted so that currents in the brain flowed posteroanteriorly at the optimal site for eliciting MEPs in FDIs, and mediolaterally for TA. Conditioning and test stimuli were given through the same coil by connecting two magnetic stimulators linked with a Bistim module (The Magstim Company, UK). At first, we determined the threshold for evoking EMG activities in the active target muscle (active motor threshold: AMT) when the subject contracted the target muscle at 5 to 10% of maximum contraction. It was defined as the lowest intensity that evoked a small response (<200 μV) compared with the prestimulus background activity. The intensity of stimulation was changed in steps of 1% of the maximum stimulator output. We used several intensities of the conditioning stimulus for each muscle in order to select the intensity for inducing maximal inhibition. The intensities used were 2 to 7% of the maximum stimulator output below the AMT, or 75 to 92% of the AMT (FDI: 85.2 ± 8.2 [mean ± SD] % for the dominant side, 89.1 ± 3.9% for the nondominant side in normal subjects and 87.1 ± 6.6% in Segawa disease; TA: 88.5 ± 9.0% in normal subjects and 85.8 ± 8.0% in Segawa disease). The test stimulus was adjusted to evoke a response as large as 0.5 mV peak to peak in the relaxed FDI and 0.3 mV in the relaxed TA when given alone. We used a randomized conditioning-test paradigm. In one session, several conditioned trials in which a test stimulus was preceded by a conditioning stimulus at various interstimulus intervals (ISIs) were randomly intermixed with control trials in which the test or conditioning stimulus was given alone. Several sessions of trials were performed to investigate the entire time course of the effect. The ISIs used were 1, 2, 3, 4, 5, and 6 msec. Ten responses were collected and averaged for each condition in which both stimuli were given, 20 responses for the control condition in which the test stimulus was given alone and 5 for the condition in which the conditioning stimulus was given alone. The experiments were done on relaxed muscles; EMG activities were monitored on an oscilloscope, and trials contaminated by EMG activities during data collection were not used for later analyses. For statistical analyses, we used the results of the session in which the maximal inhibition was elicited. For each muscle of each subject, at one ISI, we calculated a ratio of the mean amplitude of the conditioned response to that of the control response (test alone). The time course of the effect of a conditioning shock was plotted with this ratio on the ordinate and the ISI on the abscissa.

Statistical analyses. In normal subjects, we analyzed the difference in SICI between the dominant and nondominant FDIs because one previous article reported a side difference in SICI when the conditioning intensity was less than 80% AMT.[16] After we confirmed that there were no side differences in our normal subjects (Results), we pooled the data from all the patients for comparisons between the patients and normal subjects. We used a Student t-test to compare the AMT between normal subjects and patients. The mean time courses of size ratio were subjected to a two factorial analysis of variance (ANOVA) (two factors: ISI [ISI = 1, 2, 3, 4, 5, and 6 msec] and side [dominant and nondominant sides], or ISI and GROUP [normal subjects and patients]). In post hoc analyses, we used a Dunnett t-test for multiple comparisons. In this analysis, the interval was an independent variable of six levels, and the group of subjects or the side was another independent variable of two levels. The size ratio was a dependent variable. Statistical analyses were performed with SPSS 14.

For evaluating the amount of SICI for each muscle of each subject, we averaged the size ratios at ISIs of 2 to 4 msec (average size ratio [ISIs 2 to 4 msec]). We chose these ISIs because significant inhibition was obtained at ISIs of 1 to 4 msec (see Results) and the inhibition at 1 msec is known to be produced by other mechanisms than GABAergic inhibition.[17,18] We considered SICI as abnormal when the average size ratio (2 to 4 msec) significantly deviated from the normal range obtained from normal subjects (mean ± 2 SD). We compared the size ratios between the patients and normal subjects using a Student t-test. In all statistical analyses, the significance level was set at $p = 0.05$ after correction for multiple comparisons.

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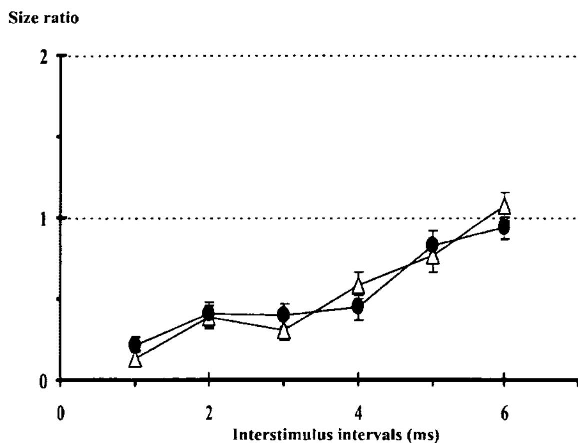

Figure 1. Mean (± SE) time courses of short-interval intracortical inhibition for first dorsal interosseous at the dominant (dots) and nondominant (triangles) sides in normal subjects. The abscissa indicates interstimulus intervals and the ordinate the amplitude ratios of conditioned motor evoked potentials (MEPs) to control MEPs. There are no significant differences between the two time courses.

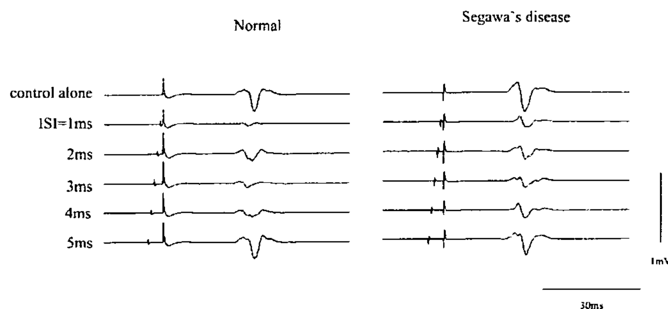

Figure 2. Typical example of motor evoked potentials (MEPs) in a normal subject (left) and a patient with Segawa disease (right). Each trace represents the averaged MEP from a relaxed first dorsal interosseous. The uppermost traces are MEPs to a test stimulus alone. The next traces are MEPs to both conditioning and test stimuli at interstimulus intervals (ISIs) of 1 to 5 msec. In the normal subject, the sizes of MEPs are decreased at ISIs of 1 to 4 msec. Similar suppression at ISIs of 1 to 4 msec is observed in the patient.

Results. *Active motor threshold.* In normal subjects, the AMT for FDI at the dominant side (mean ± SD: 37.9 ± 9.4%) was not significantly different from that at the nondominant side (37.7 ± 3.6%). The mean ± SD threshold for active FDI at more affected side was 34.4 ± 8.5% of the maximum stimulator output in patients with Segawa disease. This value did not differ from the normal value obtained from both sides (37.8 ± 7.7%) (Student *t*-test, $p > 0.05$). That for active TA muscle of the patients (37.6 ± 8.7%) was not different from that of normal subjects (42.5 ± 9%) (Student *t*-test, $p > 0.05$).

Paired pulse magnetic stimulation. We excluded the data of FDI from Patient 7 because she could not keep the FDI muscle relaxed during the experiment.

In comparison of the SICI between the dominant and nondominant sides in normal subjects (figure 1), the factor of side had no effect on the size ratio [$F(1,16) = 0.036$, $p = 0.851$], and there was no interaction between the two factors (ISI and SIDE) [$F(5,80) = 1.73$, $p = 0.136$].

In both the normal subjects and patients with Segawa disease, the inhibition was observed at ISIs of 1 to 4 msec in FDI (figures 2 and 3A). Two factorial ANOVA showed that the ISI had an effect on the size ratio [$F(5,120) = 42.4$, $p < 0.001$]. The factor of group had no effect on it [$F(1,24) = 0.015$, $p = 0.903$], and there was no interaction between the two factors (ISI and group) [$F(5,120) = 1.76$, $p = 0.127$]. These indicated that the time course for Segawa disease did not significantly differ from that for normal subjects. This was confirmed by the finding that the average size ratios (ISI 2 to 4 msec) of the patients (mean ± SD: 0.45 ± 0.16) were all within the normal range (mean ± SD: 0.42 ± 0.18, normal range 0.07 to 0.79). In the group analysis, there were no differences in the average size ratio between Segawa disease and normal subjects ($p > 0.05$, Student *t*-test) (figure 3B).

The similar inhibition was also observed in the TA muscle at ISIs of 1 to 4 msec in both groups (figure 4A). Two factors ANOVA showed that the ISI had a effect on the size ratio [$F(5,95) = 28.7$, $p < 0.01$]. The factor of group had no effect [$F(1,19) = 0.14$, $p = 0.72$], and there was no interaction between the two factors [$F(5,95) = 1.61$, $p = 1.61$]. The (mean ± SD) average size ratio (ISI = 2 to 4 msec) for normal controls was 0.49 ± 0.21 (figure 4B). The size ratio of each patient was within the normal range (0.06 to 0.91). There was no difference in the size ratio between the two groups ($p > 0.05$, Student *t*-test) (0.54 ± 0.15 for the patients).

Six out of our nine patients showed the typical clinical features. The other three adult onset patients had symptoms different from those of young onset typical patients. In the three patients with atypical clinical features (Patients 7, 8, and 9), the SICIs of both muscles were also within the normal range, even though the average size ratio of Patient 9 was biggest among all the patients (0.76 in FDI and 0.7 in TA).

Discussion. We have shown that the SICIs for both FDI and TA were normal in patients with Segawa disease, even though they had dystonia in the forearm, hand, and leg muscles. Before concluding that SICI is normal in Segawa disease, we should discuss two points: Were there any effects of levodopa treatment on the results? Since our patients included some with atypical manifestations of the disease, how would this have affected the results?

As to the first question, in patients with PD, the amount of SICI significantly decreased during the OFF period, and normalized during the ON period.[19] Segawa disease is successfully treated with levodopa and the symptoms come back several days after the cessation of levodopa.[20] To exclude the effects of levodopa, we studied our patients 18 to 32 hours after withdrawal from levodopa because these were the longest off period which the patients could tolerate, although a complete washout of levodopa would have required 1 week or more. One article[21] showed abnormal SICI after 1 day cessation of levodopa in Segawa disease. Because of these, we chose the above mentioned levodopa off periods in this experiment. It is also possible that levodopa medication may have

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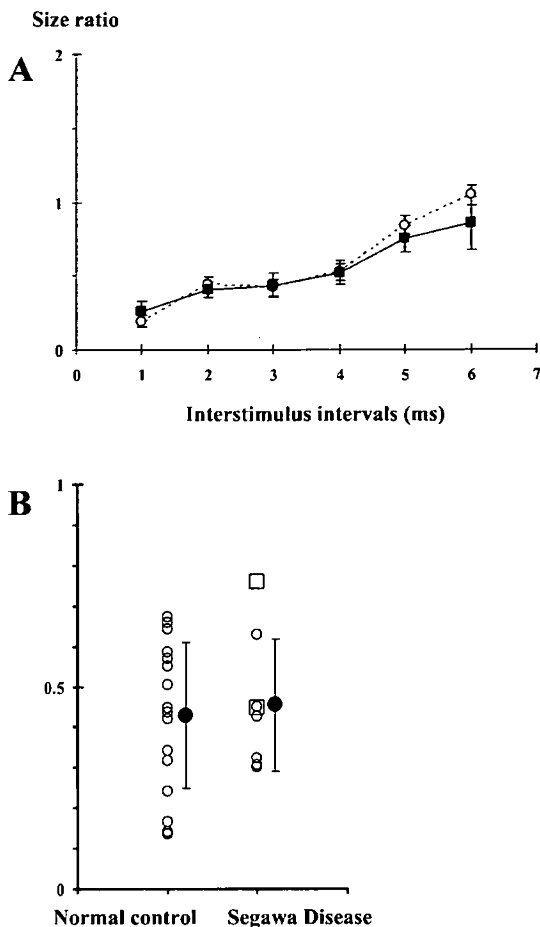

Figure 3. (A) Mean time courses of the inhibition for first dorsal interosseous (FDI) from all normal subjects (circles) and patients with Segawa disease (squares). The abscissa and ordinate are the same as figure 1. There is no significant difference between the two time courses. (B) Average size ratios (2 to 4 msec) for FDI. Dots indicate mean values of the average size ratio for each group. The bars indicate SDs, and circles the ratios for all individuals. There were no differences in the average size ratio (2 to 4 msec) between normal subjects and patients with Segawa disease including two who had not taken levodopa (squares).

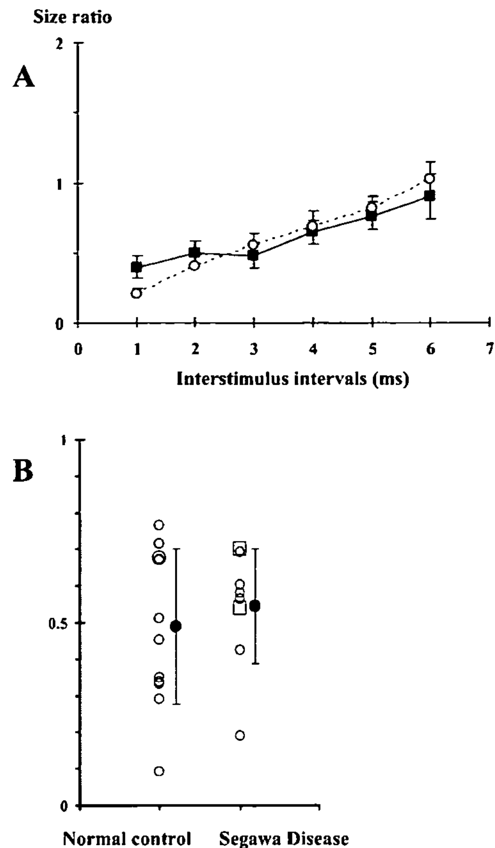

Figure 4. (A) Mean time courses of the inhibition for tibialis anterior (TA) from all normal subjects (circles) and patients with Segawa disease (squares). The abscissa and ordinate are the same as figure 1. The two time courses are not significantly different. (B) Average size ratios (2 to 4 msec) for TA. Dots indicate mean values of the average size ratio for each group. The bars indicate SDs, and circles the ratios for all individuals. In TA muscle, there were no significant differences in the average size ratio (2 to 4 msec) between the two groups of subjects including two patients who had not taken levodopa (squares).

partly normalized SICI in some of our patients. However, typical symptoms partly reappeared in the studied muscles in our patients at the time of examination, which suggests that the effect of levodopa was wearing off. Moreover, two patients had not been treated with levodopa at the time of experiments, and nevertheless SICI was normal in these patients. Therefore, we consider that the treatment solely should not explain our normal SICI.

Typically, patients with Segawa disease initially present with equinovarus appearing around 6 years of age (or younger than 10 years) and dystonia usually expands to all extremities in the second decade, mostly by 15 years of age. They also develop difficulty in hand pronation/spination and axial dystonia without torsion. The symptoms show marked diurnal fluctuation. However, various atypical symptoms have been observed in genetically proven Segawa disease patients, such as writer's cramp[22] and guitarist's cramp.[23] Six of our nine patients showed the typical clinical features. The other three adult onset patients showed atypical symptoms; the first showed coarse arm movements; the second developed rigidity, bradykinesia, and tremor similarly to PD; and the other had focal hand dystonia. The amount of SICI was normal even in the late onset, atypical patients. We consider that the SICI is normal in both the typical and atypical groups of Segawa disease. The late onset patients with atypical symptoms tended to have a larger size ratio within the normal range. Further study of more atypical patients is warranted to draw a firm conclusion about whether atypical patients have a slightly reduced SICI.

It has recently been reported that the amount of SICI in the hand muscles was abnormally decreased in patients with DYT5, even in hand muscles showing no dystonia,[21] at variance with our results. We

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showed normal SICI even in some muscles showing dystonia, especially even in TA. A possible explanation for this discrepancy is the difference in the intensity of conditioning stimulus used. They fixed the conditioning stimulus at 80% AMT. In contrast, we used several different intensities of the conditioning stimulus for each muscle in order to select the optimal intensity to elicit the maximal inhibition. The intensity relative to AMT for eliciting the maximum SICI was variable even in normal subjects (72 to 95%). Therefore, if the conditioning stimulus was fixed at a certain intensity, the amount of SICI must sometimes be smaller than the maximum inhibition. If the best intensity for SICI is more variable in the patients but clustered around 80% in normal subjects, or if the best intensity for eliciting SICI relative to AMT was different between the two groups, they may explain this discrepancy. However, neither AMT nor the best intensity for inducing SICI relative to AMT was different between the patients and normal subjects, which fails to totally explain the discrepancy. Another possible explanation is that the range of the conditioning stimulus intensity that evokes a fair amount of SICI may be narrower in the patients as compared with normal subjects. If so, the 80% AMT can evoke a fair amount of SICI in normal subjects, even if not the deepest, but only a small amount of inhibition in some of the patients. Taken as a whole, this will lead to abnormal SICI in Segawa disease. The final technical issues concern the coil used and dominance of the studied side. We used a round coil, but they used a figure of eight coil. From our experiences, we got similar amount of SICI when using either a round or a figure eight shaped coil in any subjects. This factor may not have serious influences on the results. On the other hand, previous articles have studied the dominant side in all the patients, whereas we studied the more affected side. A recent article[16] reported that the amount of SICI elicited by 80% AMT conditioning stimulus was less at the dominant side than that at the nondominant side. However, we were not able to replicate this finding. Since our results showed no side differences in the SICI in normal subjects, the side difference must not seriously influence the results, at least in our experiments.

Even though several factors may explain the discrepancy, our result that the optimal conditioning intensity evoked normal amount of SICI suggests that the motor cortical GABAergic interneurons themselves are not damaged in patients with Segawa disease even though their function may be partly affected by changes in inputs from the basal ganglia. The finding that the mean size ratio of our patients was almost the same as the mean of normal subjects in a previous report[21] supports the notion that GABAa neurons of the motor cortex are intact in Segawa disease.

The normal SICI in Segawa disease stands in striking contrast to the previously reported reduction of SICI in several movement disorders such as primary dystonia,[24] writer's cramp,[12] DYT1,[25] spasmodic torticollis,[13] and PD.[19] It is believed that the disinhibition of the motor cortex plays an important role in the generation of focal dystonia. In this case, SICI reduction is present mainly in dystonic muscles, but sometimes seen even in some muscles showing no dystonia. Moreover, in some DYT1 gene carriers, the SICI was abnormally reduced although they had no dystonia.[25] From comparison of our results with these reports, we can say that pattern of secondary disinhibition of the primary motor cortex is partly different between Segawa disease and focal dystonia. It may imply that the mechanisms for dystonia in Segawa disease are different from those for other dystonic disorders. In other words, the disinhibition (dysfunction of GABAa inhibitory interneurons) of the primary motor cortex does not contribute very much to the generation of dystonia in Segawa disease as is the case in some focal dystonias.

Neurohistochemical study of one patient with Segawa disease revealed the reduction of dopamine in the substantia nigra and striatum, and also decreased tyrosin hydroxylase protein and tyrosin hydroxylase activity in the putamen but normal in the substantia nigra.[26] The authors concluded that disturbed dopamine synthetic capacity or a reduced arborization of striatal dopamine terminals might be the major disturbance in Segawa disease. The sleep and saccadic eye movement studies in patients with Segawa disease have also suggested the dysfunction of the nigrostriatal DA neurons.[4] However, such dysfunction of dopamine systems has not been proven in other dystonic disorders. These results also support our proposal that the pathomechanisms for dystonia in Segawa disease are partly different from those for other dystonias.

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Origin of facilitation in repetitive, 1.5 ms interval, paired pulse transcranial magnetic stimulation (rPPS) of the human motor cortex

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Abstract

Objective: Repetitive paired-pulse TMS (rPPS) given at an interstimulus interval (ISI) of 1.5 ms has been reported to induce a lasting motor evoked potential (MEP) facilitation. This after-effect was considered to be a cortical event because F-waves were not affected by the same rPPS. To confirm its cortical facilitation, we compared the after-effects of rPPS on MEPs to single pulse TMS over the motor cortex (motor cortical MEPs) with those to brainstem stimulation (brainstem MEPs).
Methods: Subjects were 10 healthy volunteers. Suprathreshold paired-pulse TMS at an ISI of 1.5 ms was applied to the motor cortex for 30 min at a rate of 0.2 Hz. After intervention, we measured motor cortical MEPs for 30 min. We also studied brainstem MEPs in five subjects.
Results: Motor cortical MEPs were facilitated to about 190% of baseline ($p < 0.001$) for 10 min post rPPS intervention and returned to the baseline at 10–15 min post intervention. Brainstem MEPs were not affected by the intervention.
Conclusions: The facilitation of MEPs after rPPS at an interval of 1.5 ms occurs at the motor cortex.
Significance: rPPS at an interval of 1.5 ms is an effective method for increasing motor cortical excitability.
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Keywords: Repetitive transcranial magnetic stimulation (rTMS); Paired pulse stimulation; Motor cortex; Monophasic pulse; I wave periodicity

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS) protocols have been reported to be a potential tool to modify human motor cortical excitability. High frequency rTMS tends to induce facilitatory after-effects and low frequency rTMS inhibitory effects (Chen et al., 1997; Maeda et al., 2000a,b; Sommer et al., 2002), although there is a moderate interindividual variability in the direction of effect (Maeda et al., 2000a). So far, most of the studies have used biphasic rTMS (Berardelli et al., 1998, 1999; Chen et al., 1997; Di Lazzaro et al., 2002; Huang et al., 2005; Maeda et al., 2000a,b; Pascual-Leone et al., 1994; Romeo et al., 2000).

We have previously shown that monophasic TMS has a stronger short-term effect during repetitive stimulation than biphasic TMS (Arai et al., 2005). This finding suggests that monophasic pulses preferentially activate population of neurons oriented in the same direction so that their effects readily summate. In the same way, it is possible that monophasic rTMS would result in a more powerful after-effect than biphasic rTMS. Indeed, Thickbroom and coworkers (2006) produced a powerful after-effect by repet-

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itive, monophasic, paired pulses TMS delivered at an I-wave periodicity (the rPPS protocol). rPPS with TMS pulses paired at 1.5 ms for 30 min facilitated motor evoked potentials (MEPs) to a single TMS delivered over the motor cortex (motor cortical MEPs), which lasted up to 10 min after the cessation of the intervention. This facilitation was proposed to occur at the cortical level because F-waves were not affected by the same protocol. However, since F-waves can reflect the activity of only a small portion of spinal motor neurons, we cannot be sure whether the whole spinal cord excitability was indeed unchanged. In contrast, MEPs to brainstem stimulation (brainstem MEPs) are considered to reflect the excitability of a larger population of spinal motor neurons than do F-waves, because it can activate most of the motor neurons in the spinal cord. In this communication, we aimed to differentiate between the cortical and subcortical loci of facilitation by comparing the after-effects of rPPS between motor cortical and brainstem MEPs. Determination of the site of action would have an important implication for the future application of this novel protocol for treating neurological disorders.

2. Subjects and methods

2.1. Subjects

Subjects were 10 healthy volunteers (2 women, 8 men, mean age ± SD: 38.0 ± 5.3 years) who gave their informed consent to participate in the experiments. None of the subjects had neurological, psychiatric or other medical problems or had any contraindication to TMS (Wassermann, 1998). The protocol was approved by the Ethics Committee of the University of Tokyo and was carried out in accordance with the ethical standards of the Declaration of Helsinki.

2.2. Stimulation and recordings

Subjects were seated on a reclining chair and motor evoked potentials (MEPs) were recorded from the right first dorsal interosseus muscle (FDI) (dominant hand in all subjects). Pairs of Ag/AgCl surface cup electrodes (9 mm in diameter) were placed over the muscle belly (active electrode) and the metacarpophalangeal joint of the index finger (reference electrode). Responses were amplified with an amplifier (Biotop, GE Marquette Medical Systems, Japan) through filters set at 100 Hz and 3 kHz, digitized with A/D converter at a sampling rate of 20 kHz and stored in a computer (TMS bistim tester, Medical Try System, Japan) for later offline analysis.

Transcranial magnetic stimulation (TMS) was given over the hand area of the left primary motor cortex (M1) at a position optimal for eliciting MEPs in the right FDI with a figure of eight coil (external diameter at each wing 9 cm, Magstim Co., Whitland, Dyfed, UK). The coil was held tangential to the scalp with the handle pointing backwards at about 45° laterally, that is perpendicular to the central sulcus. According to the previous study (Sakai et al., 1997), this is the optimal orientation for activating the corticospinal system trans-synaptically via horizontal cortical connections. To determine the optimal site for FDI, we stimulated several positions separated by 1 cm with the same intensity and defined the hot spot as the site where the largest responses were elicited. The position was marked with a red pen on the scalp for repositioning the coil at the same site throughout the experiments.

Single pulse TMS was delivered by a Magstim 200 magnetic stimulator (Magstim Co., Whitland, Dyfed, UK), and paired pulse stimuli were delivered by two Magstim 200 stimulators connected with a Bistim module (Magstim Co., Whitland, Dyfed, UK).

For sham stimulation (see below), surface cup electrodes were placed over the vertex (Cz of international 10–20 system) and the left-hand motor area in each subject. An electric pulse of 0.2 ms duration was delivered through those electrodes with a conventional electric stimulator. The intensity was fixed at two times the sensory threshold for skin sensation.

Brainstem (BST) electrical stimulation was performed to evaluate spinal motoneuronal excitability changes because MEPs to this stimulation must reflect activity of most of spinal motor neurons (Ugawa et al., 1991). Anode (right) and cathode (left) were attached over the mastoid processes. Stimulation was given with a high voltage electric stimulator (D180A: 0.1 ms duration, maximal output 1.2 A, 1200 V; Digitimer, Welwyn Garden City, UK).

2.3. Interventions

2.3.1. rPPS intervention

For rPPS intervention (rPPS 30 in Fig. 1), we used the same protocol as that reported by Thickbroom et al. (2006). Paired stimuli of equal strength were delivered at an interval of 1.5 ms. The stimulus intensity was set to elicit MEPs of 0.3–0.5 mV when delivered as a pair. Paired stimuli were applied over the left hand motor area every 5 s, and 360 paired stimuli were administered in total for 30 min. During intervention, 360 MEPs (each one MEP elicited by one paired TMS) were obtained and a series of 60 MEP amplitudes were averaged to obtain the mean MEP amplitudes at 5 min intervals. Even in real rPPS, two electrodes were fixed at the same positions over the scalp as those in the sham stimulation (see the next paragraph). No currents were in real rPPS.

2.3.2. Realistic sham intervention

In sham intervention (sham 30 in Fig. 1), we performed realistic sham stimulation described previously (Okabe et al., 2003). Paired electric stimuli were given every 5 s for 30 min. The coil was not connected to a stimulator and placed over left M1. Another coil was placed near the subject. This coil was connected to Bistim module combining two Magstim 200 stimulators. These stimulators

were discharged simultaneously with the electric stimuli to produce the same sound as that associated with real rPPS intervention. The simulation parameters of paired stimuli were the same as those used in a real rPPS intervention.

2.4. Motor cortical MEPs

Motor cortical excitability was assessed by measuring the peak-to-peak amplitude of MEPs from the right FDI to single pulse TMS (motor cortical MEPs). During the experiments, subjects were encouraged to be fully relaxed with the aid of an oscilloscope monitor of the target EMG. The stimulus intensity of single pulse TMS was adjusted to elicit MEPs approximately 0.3 mV in the baseline condition. Trials contaminated with voluntary EMG activities were discarded from analysis.

2.5. Brainstem MEPs

For BST electrical stimulation, the intensity was set to produce brainstem MEPs of 0.2–0.3 mV similar sized to motor cortical MEPs. Relaxation of FDI was monitored with the aid of an oscilloscope monitor. The used intensity was 30–45% of the maximal electrical stimulator output (the highest used stimulus intensity: 0.1 ms duration, 45% of 1.2 A, 1200 V).

2.6. Timelines of experiments (Fig. 1)

Four different experiments were performed. In the same subject, two successive experiments were separated by at shortest one week interval. The order of experiments was randomized between subjects. Timelines of all the experiment are shown in Fig. 1.

Experiment (1) rPPS-TMS. Ten subjects participated in this experiment. Before and after rPPS intervention (rPPS 30), motor cortical excitability was measured by single pulse TMS. The time points of motor cortical MEP measurements are shown in Fig. 1(b, P1–6). At time point B, to evaluate the baseline motor cortical excitability, 20 motor cortical MEPs were collected every 15 s. After rPPS intervention, 120 motor cortical MEPs were collected in total for 30 min (P1–6). TMS were applied every 15 s, and mean MEP amplitudes were obtained from 20 motor cortical MEPs every 5 min.

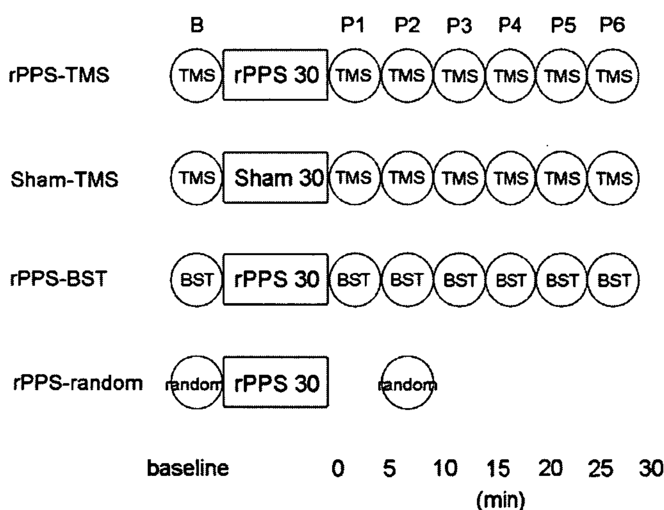

Fig. 1. Timelines of our experiments (see Section 2).

Experiment (2) sham-TMS. Four subjects participated in this study. Before and after sham intervention (sham 30), motor cortical MEPs were measured. The time points were the same as those in experiment 1.

Experiment (3) rPPS-BST. To clarify the origins of facilitation after rPPS intervention, we performed BST electrical stimulation (Ugawa et al., 1991) in five subjects. Before rPPS intervention, 8 brainstem MEPs were obtained every 15 s to measure the baseline size of brainstem MEP. The protocol of rPPS intervention was the same as that of experiment 1 (rPPS 30). After rPPS intervention, at each time point (P1–6), BST stimuli were given every 15 s and 8 brainstem MEPs were collected. Thus, 48 brainstem MEPs were collected in total for 30 min (P1–6). Mean MEP amplitudes were obtained from 8 brainstem MEPs at each time point.

Experiment (4) rPPS-random. To exclude the effect of prediction of uncomfortable stimulation, we also performed another experiment in 3 subjects. Before rPPS intervention (B random in Fig. 1), 8 motor cortical MEPs and 8 brainstem MEPs were obtained in a random order (i.e., 16 stimuli were given every 15 s in a random order). The protocol of rPPS intervention was the same as that of experiment 1. After 5 min post intervention (P2 random in Fig. 1), 8 motor cortical MEPs and 8 brainstem MEPs were obtained in a random order.

2.7. Data analysis and statistics

The effect of different conditions (rPPS-TMS, rPPS-BST) on the MEP size during rPPS intervention was studied using two way repeated measures analysis of variance (rANOVA) (between group factor; condition, within subject factor; time). The effect of different conditions (rPPS-TMS, rPPS-BST, Sham-TMS) on the time course of MEP size after intervention was evaluated with two way rANOVA (between group factor; condition, within subject factor; time). For each condition, the effect of the intervention on the time course of MEP size was analyzed using one-way factorial ANOVA. The Greenhouse–Geisser correction was used if necessary to correct for nonsphericity. Post hoc Bonferroni method was employed for further analysis. For experiment 4 (rPPS-random), MEP amplitudes were compared using paired t test. P values less than 0.05 were considered to be significant. All figures represent group data. Data are expressed as means $\pm$ SE except Fig. 4 (see Section 3). Data were analyzed using SPSS for Windows version 13.0.

3. Results

None of the subjects reported any adverse effects during and after any interventions.

3.1. rPPS intervention

Fig. 2a shows typical example of MEPs during rPPS intervention in one subject. During rPPS intervention, each response is an average MEP made from 60 raw MEPs for 5 min. Fig. 2b shows the mean MEP amplitude at 5 min interval during 30 min of rPPS intervention. In both experiments (rPPS-TMS and rPPS-BST), MEPs were increased similarly. Two way rANOVA revealed neither significant effect of the condition ($F[1,13] = 0.034$, $p > 0.05$), the time ($F[2.335,30.354] = 1.233$, $p > 0.05$) nor significant interaction between the condition and time ($F[2.335,30.354] = 0.159$, $p > 0.05$). For rPPS-TMS or rPPS-BST, one way factorial ANOVA revealed no significant effects of the time ($F[5,45] = 0.525$, $p > 0.05$ for rPPS-TMS, and $F[5,20] = 2.081$, $p > 0.05$ for rPPS-BST).

3.2. After-effect of rPPS or sham intervention

The upper trace of Fig. 3a shows typical motor cortical MEPs before and after rPPS intervention. Average MEPs every 5 min are depicted. The robust motor cortical MEP facilitation was seen for 10 min after intervention, and they returned to the baseline at 10–15 min post intervention. The lower trace of Fig. 3a shows typical brainstem MEPs. No facilitation was elicited in brainstem MEPs after intervention.

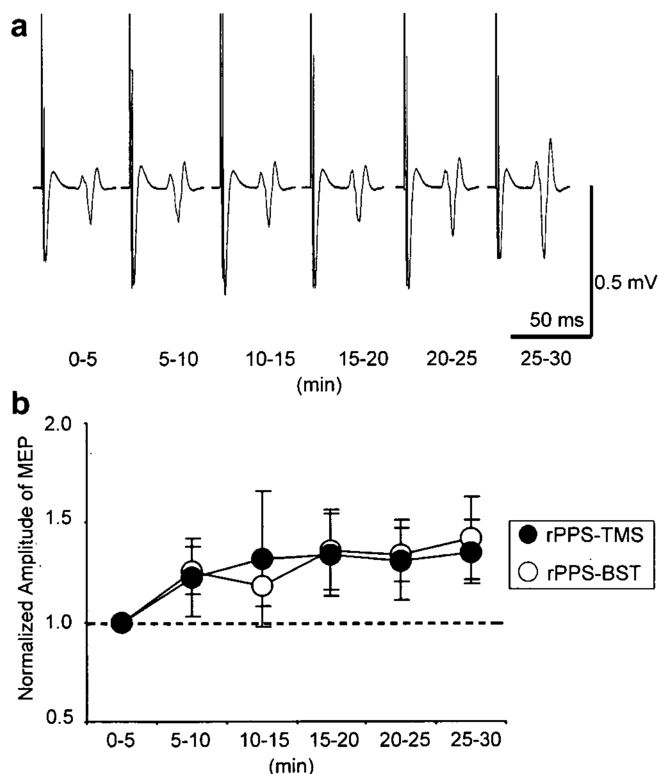

Fig. 2. MEPs during rPPS intervention. (a) Typical example of MEP waveforms from one subject. (b) Group mean MEP amplitude during rPPS intervention. MEPs are normalized to the mean of MEP amplitudes measured 5 min after the intervention. We observed similar MEP facilitation during rPPS intervention in both rPPS-TMS and rPPS-BST experiments. All data are means ± SEs.

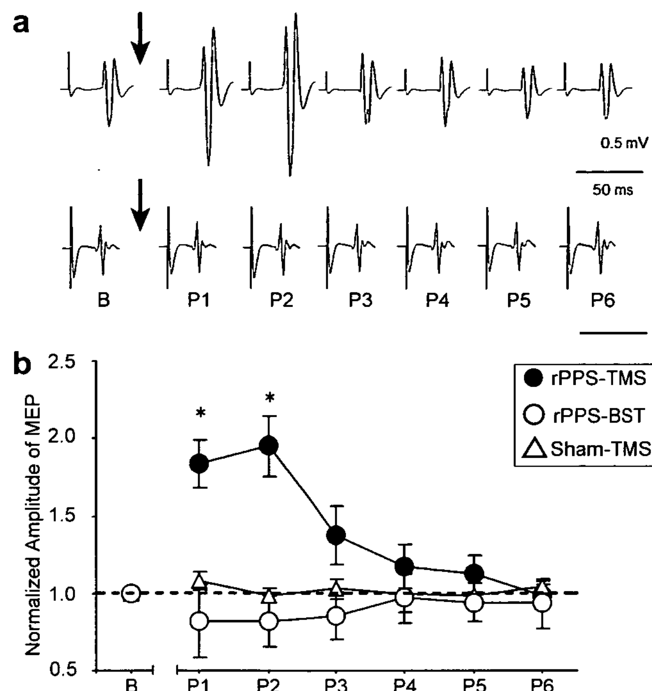

Fig. 3. After-effects of rPPS and sham intervention. (a) Representative example of MEPs before and after rPPS intervention. The arrows show the timing of rPPS intervention. Upper trace shows motor cortical MEPs (elicited by TMS) before and after rPPS intervention. Lower trace shows brainstem MEPs (elicited by BST stimulation). Only MEPs to TMS (motor cortical MEPs) were facilitated after rPPS intervention. (b) Time courses of the MEP amplitude following rPPS or sham intervention. MEPs were normalized to mean MEP amplitude measured at baseline. The facilitation was seen at P1 and P2 period in motor cortical MEPs after the real rPPS. Asterisks, $p < 0.001$.

Fig. 3b shows time courses of the MEP amplitude for three different conditions (rPPS-TMS, rPPS-BST, Sham-TMS). Two way rANOVA revealed a significant effect of the condition ($F[2,16] = 5.610$, $p = 0.014$) and a significant interaction between the condition and time ($F[5.954, 47.630] = 4.765$, $p = 0.001$), but no significant effect of the time ($F[2.977,47.630] = 1.700$, $p = 0.129$). For rPPS-TMS, subsequent one way factorial ANOVA revealed a significant effect of the time ($F[6,54] = 9.925$, $p < 0.001$). Post hoc analysis revealed that the motor cortical MEPs were significantly larger than the baseline (B) for 10 min after the end of the intervention (at time point P1; $p < 0.001$, P2; $p < 0.001$, P3–P6; $p > 0.05$). For rPPS-BST and Sham-TMS, one way factorial ANOVA did not reveal significant effects of the time ($F[6,24] = 0.586$, $p > 0.05$, and $F[6,18] = 0.394$, $p > 0.05$).

Fig. 4 shows mean ± SD MEP sizes before and 5 min after the intervention. Following rPPS intervention, MEP amplitudes to TMS were enhanced (mean ± SD; baseline:

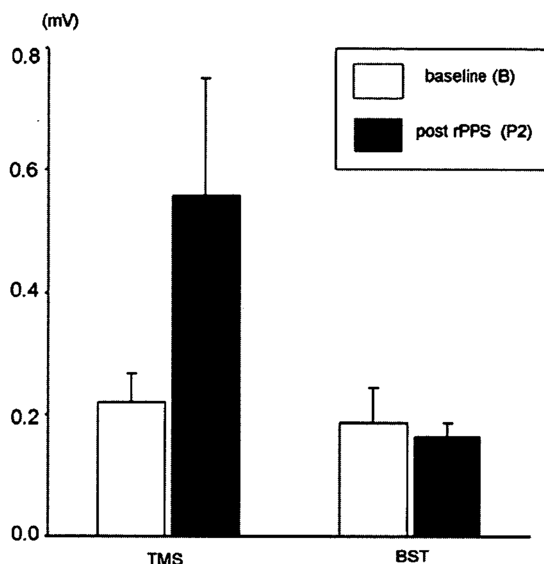

Fig. 4. Bars show mean amplitudes of cortical and brainstem MEPs. The timing of evaluation was 5 min post intervention. All data are means ± SDs. The facilitation was elicited on the motor cortical MEPs whereas brainstem MEPs were unaffected.

0.22 ± 0.05 mV, post 5 min: 0.56 ± 0.19 mV; $p < 0.05$, paired t test), while those to BST stimulation did not change significantly (baseline: 0.19 ± 0.05 mV, post 5 min: 0.16 ± 0.02 mV; $p > 0.05$, paired t test).

4. Discussion

Our main finding of the present study is that motor cortical MEPs were enhanced for 10 min after the cessation of rPPS intervention whereas there were no changes in brainstem MEPs. Whereas the size of responses to TMS reflects the excitability of the motor system as a whole, brainstem MEPs chiefly reflect the excitability of the motor system caudal to the brainstem including the spinal cord. Therefore, the facilitation should occur at the cortical level in agreement with the results of Thickbroom and colleagues (2006). We not only replicated their results but also provided robust support for the cortical site of action, an important information for the newly developed intervention protocol if it is to be used in the future for the treatment of neurological disorders.

Our results are at variance with those by Thickbroom et al. (2006) in that we did not observe marked facilitation of MEPs to paired TMS during rPPS intervention, although considerable facilitatory after-effects of MEPs to single pulse TMS were seen. A possible explanation for this discrepancy is that different populations of descending volleys contribute to MEP generation in single and paired pulse TMS. Thus, even though the same sizes of MEP are induced by the two methods, the volleys elicited by paired stimulation may not be enhanced but those by single pulse TMS are. In any case, single pulse TMS should be used for probing motor cortical excitability correctly.

Although the duration of motor cortical MEP facilitation after rPPS intervention was similar to those of Thickbroom et al. (2006), there was some difference in the magnitude of facilitation between two studies. There are at least two possible reasons for this discrepancy. The first is the interindividual variability. In our results, the standard deviation (variability) of MEP size at 5–10 min post rPPS intervention was 61.8% (mean percentage changes of MEP size = 195.40%), comparable with the variability previously reported by Maeda et al. (mean = 137.87%, SD = 53.59% after 10 Hz rTMS) (Maeda et al., 2000a). In contrast, the previous report by Thickbroom et al. (2006) showed a larger variability (mean = 476%, SD = 381% at 6 min post intervention). These data indicate that our effect was steadier even though the amount of MEP facilitation was smaller. In addition, the difference in studied subjects, perhaps in race, may explain the different magnitude of facilitation after rPPS intervention.

In contrast to its magnitude, the time course of after-effect by rPPS was very similar to what has been described previously (Thickbroom et al., 2006). The present result indicates that the after-effect of rPPS intervention is quite consistent in duration. This message is an important point when using it as a treatment of neurological disorders, because it is the lasting after-effect of rTMS that mainly produces the therapeutic effect.

4.1. Possible mechanisms of facilitation induced by rPPS

The fact that the resting motor threshold (RMT) did not change after rPPS (Thickbroom et al., 2006) suggests that the membrane excitability of pyramidal output cells was not affected by rPPS (Ziemann et al., 1996). As well as membrane excitability, the excitability of a part of synapses between interneurons and cortico-spinal cells has an influence on RMT (Di Lazzaro et al., 2003). Such parts of synapses may also not be affected by rPPS. In spite of no influence on RMT, rPPS intervention made the recruitment curves steeper after intervention (Thickbroom et al., 2006). This suggests an enhancement of efficacy of some synapses which contribute to generation of MEPs to single pulse TMS but do not much contribute to determine the threshold (mainly contribute to small sized MEPs). The previous authors speculated that repeated activation of facilitatory I wave interaction (Tokimura et al., 1996; Hanajima et al., 2002; Ziemann et al., 1998) may reinforce synaptic efficacy and the facilitation induced by rPPS may be a consequence of modulation of the trans-synaptic events. Alternatively, single pulse TMS not only activates neurons oriented in a single direction preferentially but may also activate other neurons oriented in other directions subliminally. These subliminally activated neurons might get more responsive to single pulse TMS after rPPS intervention, leading to the facilitation of MEPs.

Although further studies are needed to clarify the precise mechanism for the facilitatory effect induced by rPPS intervention, rPPS intervention protocol is one of promising

stimulation methods for increasing motor cortical excitability.

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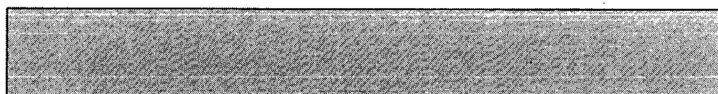

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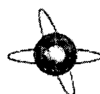

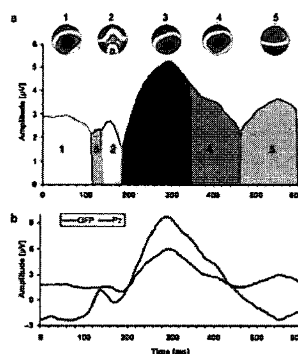

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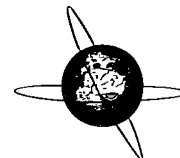

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Quadro-pulse stimulation is more effective than paired-pulse stimulation for plasticity induction of the human motor cortex

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Abstract

Objective: Repetitive paired-pulse transcranial magnetic stimulation (TMS) at I-wave periodicity has been shown to induce a motor-evoked potential (MEP) facilitation. We hypothesized that a greater enhancement of motor cortical excitability is provoked by increasing the number of pulses per train beyond those by paired-pulse stimulation (PPS).

Methods: We explored motor cortical excitability changes induced by repetitive application of trains of four monophasic magnetic pulses (quadro-pulse stimulation: QPS) at 1.5-ms intervals, repeated every 5 s over the motor cortex projecting to the hand muscles. The after-effects of QPS were evaluated with MEPs to a single-pulse TMS, motor threshold (MT), and responses to brain-stem stimulation. These effects were compared to those after PPS. To evaluate the QPS safety, we also studied the spread of excitation and after discharge using surface electromyograms (EMGs) of hand and arm muscles.

Results: Sizes of MEPs from the hand muscle were enhanced for longer than 75 min after QPS; they reverted to the baseline at 90 min. Responses to brain-stem stimulation from the hand muscle and cortical MEPs from the forearm muscle were unchanged after QPS over the hand motor area. MT was unaffected by QPS. No spreads of excitation were detected after QPS. The appearance rate of after discharges during QPS was not different from that during sham stimulation.

Conclusions: Results show that QPS can safely induce long-lasting, topographically specific enhancement of motor cortical excitability.

Significance: QPS is more effective than PPS for inducing motor cortical plasticity.

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Keywords: Repetitive transcranial magnetic stimulation; LTP; Motor cortex

1. Introduction

Repetitive transcranial magnetic stimulation (rTMS), a noninvasive method to activate cortical neurons focally and transsynaptically in the human brain, has the potential to modulate motor cortical excitability. During the past decade, high-frequency rTMS has been shown to induce facilitatory effects on the human motor cortex (Pascual-Leone et al., 1994; Chen et al., 1997b; Berardelli et al., 1998; Maeda et al., 2000; Baumer et al., 2003; Peinemann et al., 2004). Other methods using direct current (transcranial direct current stimulation, tDC; Nitsche and Paulus, 2001), ischemic nerve block (INB; Ziemann et al., 1998), paired associative stimulation (PAS; Stefan et al., 2000; Wolters et al., 2003; Quartarone et al., 2006), and theta-burst stimulation (TBS; Huang et al., 2005) have also been shown to induce long-lasting aftereffects. Thickbroom et al. (2006) showed recently that repetitive, monophasic paired-pulse rTMS (paired-pulse stimulation; PPS) at 1.5-ms

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interval induces motor cortical excitability enhancement lasting up to 10 min after cessation of the intervention. In our previous investigation, the aftereffects of PPS continued as long as those described by Thickbroom et al. (2006) (Hamada et al., 2007), although their effects were smaller than those of Thickbroom et al. (2006). A few reports have shown that the site of action of PPS is the motor cortex (Hamada et al., 2007; Di Lazzaro et al., 2007).

The mechanism of these aftereffects is suggested to be synaptic modification similar to that of long-term potentiation (LTP) or long-term depression (LTD) in animal experiments (Chen et al., 1997b; Ziemann et al., 1998; Stefan et al., 2000; Siebner et al., 2000; Wolters et al., 2003; Lee et al., 2003; Huang et al., 2005; Quartarone et al., 2006; Cooke and Bliss, 2006). Thickbroom et al. (2006) speculated that PPS might reinforce transsynaptic events within the motor cortex because 1.5 ms corresponds to I-wave periodicity. This idea was also supported by results of paired-pulse TMS (Tokimura et al., 1996; Hanajima et al., 2002) and quadro-pulse TMS (Amassian and Deletis, 1999). The lack of invasive recording of synaptic responses in conscious humans renders any inference of precise neuronal mechanisms underlying these aftereffects speculative (Cooke and Bliss, 2006), but scientific knowledge of synaptic plasticity can provide important information for plasticity induction in the human motor cortex (Cooke and Bliss, 2006).

In animal experiments, the threshold for LTP induction is a complex function of the intensity and pattern of tetanic stimulation (Malenka, 1991; Bliss and Collingridge, 1993). The number of pulses per train is known to be a very potent factor of tetanic stimulation to influence the level of synaptic plasticity in the hippocampus (Nakao et al., 2004). These lines of evidence lead us to presume that a greater enhancement of motor cortical excitability can be provoked by increasing the number of pulses per train. The present study investigated the effects of repetitive application of four monophasic magnetic pulses (quadro-pulse stimulation, QPS) on motor cortical excitability.

Unfortunately, no safety guidelines for complex rTMS protocols such as QPS, PPS (Thickbroom et al., 2006), or TBS (Huang et al., 2005) exist. According to established guidelines of rTMS (Chen et al., 1997c; Wassermann, 1998), seizure occurrence, post-TMS electromyogram (EMG) activity, and the spread of excitation (SE) to proximal muscles are considered unsafe. Post-TMS EMG activity referred to continuation of EMG activity after cessation of rTMS; it was thought to be a possible correlate to an after discharge. In addition, SE was thought to indicate a breakdown of lateral inhibition in the cortex (Pascual-Leone et al., 1993, 1994). For these reasons, we have also studied the safety of QPS by monitoring SE or occurrence of post-TMS EMG activity after QPS. This information will facilitate development of further clinical applications of QPS.

2. Subjects and methods

2.1. Subjects

Subjects were 16 healthy volunteers (3 women, 13 men; mean $\pm$ SD age, 37.0 ± 6.0) who gave their written informed consent to participate in the experiments. No subjects had neurological, psychiatric disorders or other medical problems or had any contraindication to TMS (Wassermann, 1998). All were right-handed according to the Oldfield handedness inventory (Oldfield, 1971). The protocol was approved by the Ethics Committee of the University of Tokyo and was carried out in accordance with the ethical standards of the Declaration of Helsinki.

2.2. Recordings

Subjects were seated on a comfortable chair and told to keep the target muscle relaxed. Motor-evoked potentials (MEPs) were recorded from the right first dorsal interosseous (FDI) muscle. Pairs of Ag/AgCl surface electrodes (9 mm diameter) were placed over the muscle belly (active electrode) and over the metacarpophalangeal joint of the index finger (reference). Responses were amplified (Biotop; GE Marquette Medical Systems, Japan) through filters set at 100 Hz and 3 kHz, digitized with an A/D converter at a sampling rate of 20 kHz, and then stored on a computer for offline analysis (TMS bistim tester; Medical Try System, Japan).

2.3. Stimulation

Transcranial magnetic stimulation (TMS) was administered through a figure-of-eight coil (9 cm external diameter at each wing; The Magstim Co. Ltd., Whitland, Dyfed, UK). Single monophasic TMS pulses were delivered using a magnetic stimulator (Magstim 200; The Magstim Co. Ltd.). Paired- or quadro-pulse stimuli were delivered by two or four stimulators (Magstim 200^2; The Magstim Co. Ltd.) connected by a specially designed combining module (The Magstim Co. Ltd.). This device combines the outputs from four stimulators to allow a train of 2–4 monophasic magnetic pulses to be delivered through a single coil.

The coil was placed tangentially over the scalp to induce currents in the brain flowing at about 45 deg in an anterior-medial direction, which is almost perpendicular to the central sulcus. This was the optimal orientation for activating the corticospinal tracts transsynaptically via horizontal cortical connections (Sakai et al., 1997).

The optimal site for eliciting MEPs in the right FDI muscle (i.e., the *hot spot*) was first determined before each experiment. We stimulated several positions 1 cm apart from each other using the same intensity. The *hot spot* was defined as the site where the largest responses were elicited. The position was marked using a red pen on the scalp for repositioning the coil. Then we determined the thresholds. The resting motor threshold (RMT) was defined as

the lowest intensity that evoked a response of at least 50 μV in the relaxed FDI in at least five of ten consecutive trials (Rossini et al., 1994). The active motor threshold (AMT) was defined as the lowest intensity that evoked a small response (>100 μV), when the subjects maintained a slight contraction of the right FDI (5–10% of the maximum voluntary contraction) with the aid of an oscilloscope monitor in more than five of ten consecutive trials. The stimulus intensity was changed in steps of 1% of the maximum stimulator output of each magnetic stimulator.

Brain-stem electrical stimulation was performed using a method described in a previous study (Ugawa et al., 1991). The anode (right) and cathode (left) were attached to the skin overlying the mastoids. Stimulation was performed using a high-voltage electrical stimulator (maximal output 1.2 A, 1200 V, D180A; Digitimer Ltd., Welwyn Garden City, UK).

2.4. Conditioning

2.4.1. Quadro-pulse stimulation (QPS) and paired-pulse stimulation (PPS)

Quadro-pulse stimulation (QPS) and paired-pulse stimulation (PPS) were applied over the *hot spot* for FDI. One train consisted of four pulses at the same intensity separated by 1.5 ms, repeatedly given at 0.2 Hz in QPS; it consisted of paired pulses of equal intensity separated by 1.5 ms, repeatedly given at 0.2 Hz in PPS. Because there was no aftereffect of single-pulse rTMS at 0.2 Hz (Thickbroom et al., 2006), we did not perform this conditioning.

2.4.2. Realistic sham conditioning

Realistic sham conditioning was described previously (Okabe et al., 2003). Four electric pulses (each electric pulse: duration, 0.2 ms; intensity, twice sensory threshold) were given to the skin of the head at 0.2 Hz using a conventional electric peripheral nerve stimulator to mimic the skin sensation of TMS. Electric pulses were applied through the electrodes placed over the left-hand motor area and Fz. A coil, which was disconnected with the stimulator, was placed over the left-hand motor area for mimicking real TMS. Another coil, which was connected to a combining module with four Magstim 200^2 stimulators, was held off the scalp but placed near the subject; it was discharged simultaneously with the scalp electrical stimulation to produce a similar sound to that associated with real QPS.

2.5. Timelines of experiments and measurement parameters (Fig. 1)

Seven different experiments were performed. For each subject, two successive experiments were separated by at least a one-week interval. The order of experiments was pseudorandomized and counterbalanced among subjects. Timelines of all experiments, except for the one studying safety, are shown in Fig. 1. We defined "0" min as the end of each conditioning (Fig. 1).

2.5.1. Experiment 1: Comparison between QPS and PPS with the same number of trains

In this experiment, 12 subjects participated. To compare the effects of QPS with those of PPS, 360 trains of QPS, PPS, or sham stimulation were applied at 0.2 Hz for 30 min. The stimulation intensity was set to elicit MEPs as large as 0.4 mV by each train of quadro pulses or paired pulses. The mean (±SD) stimulus intensity of each pulse was 130 ± 24% AMT (82 ± 7% RMT; 74–98% RMT) in QPS conditioning; the mean intensity of each pulse was 148 ± 27% AMT (98 ± 10% RMT) in PPS conditioning. These characteristic conditioning protocols are designated as QPS-supra-360 and PPS-supra-360 for subsequent discussion. We also performed sham conditioning for 30 min (QPS-sham-360) in four subjects.

2.5.1.1. MEPs recorded from FDI muscle during conditioning. Conditioning was started from "−30" min (Fig. 1). During QPS-supra-360 and PPS-supra-360, we obtained a total of 360 MEPs (each MEP elicited by one train of quadro pulses or paired pulses every 5 s). The peak-to-peak amplitude of each MEP was measured. Every 60 MEP responses were averaged to obtain the mean MEP amplitudes at 5-min intervals. During the experiments, EMG activity of the FDI was monitored using an oscilloscope monitor. Trials contaminated with voluntary EMG activity were discarded from analyses.

2.5.1.2. MEPs recorded from FDI muscle before and after conditioning. The aftereffects of QPS-supra-360, PPS-supra-360, or QPS-sham-360 were evaluated according to changes in the peak-to-peak amplitude of MEPs to single-pulse TMS (cortical MEPs) in the right FDI muscle at rest. In all, 20 cortical MEPs were collected every 14.5–15.5 s before conditioning and during six epochs following each conditioning: 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 min. We used this long interval to match that of brain-stem electrical stimulation (see below; Experiment 5). The stimulus intensity of single-pulse TMS was adjusted to elicit MEP of approximately 0.4 mV before conditioning and was kept constant during all experiments. The excitability of each period was estimated by averaging 20 MEPs; it was expressed as the size ratio relative to the baseline average MEP.

2.5.2. Experiment 2: Effects of the intensity and the number of trains of QPS on aftereffects

In this experiment, 12 subjects participated. Two subjects not included in Experiment 1 were also enrolled. We performed three conditioning types to elucidate the relative contribution of intensity and number of trains to the QPS aftereffects: (1) QPS-sub-360, 360 trains of QPS with 90% AMT (each pulse at 90% AMT); (2) QPS-supra-180, 180 trains of QPS with 130% AMT; and (3) QPS-sub-180, 180 trains of QPS with 90% AMT. During QPS-sub-360 and QPS-sub-180 conditioning, we were unable to obtain any MEPs to one train of quadro pulses. Before and after

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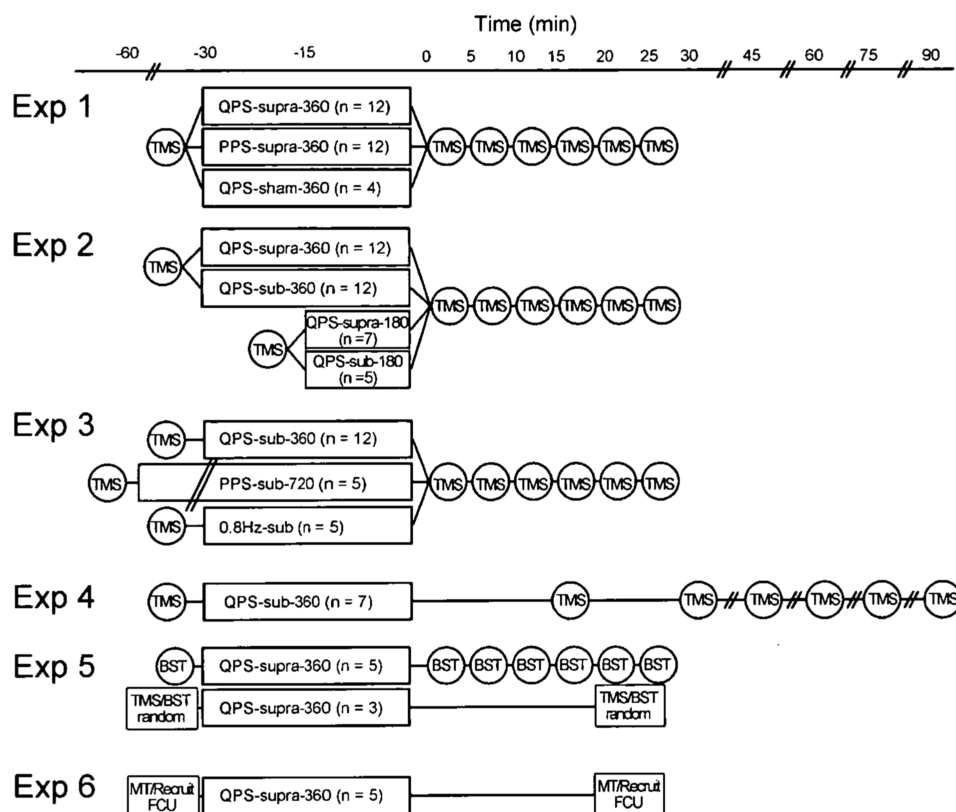

Fig. 1. Timelines of experiments (see Section 2.5).

each conditioning, cortical MEPs were recorded in the same manner as that described for Experiment 1. Results of QPS-supra-360 obtained in Experiment 1 were also used for comparison with those of Experiment 2.

2.5.3. Experiment 3: Comparison between QPS and PPS with the same total number of pulses

Five subjects participated in this experiment. One subject, who was not included in Experiments 1 and 2, was enrolled. Because we should consider the effects of the total number of pulses when we compare the aftereffects of QPS to those of PPS, we performed the following conditioning protocols with the same total number of pulses as that of QPS-sub-360 (1440 pulses): (1) PPS-sub-720, 720 trains of PPS with 90% AMT and (2) 0.8 Hz-sub, continuous single-pulse TMS at 0.8 Hz with 90% AMT for 30 min. The latter one (0.8 Hz-sub) contains the same number of pulses with QPS in 1 s. During these conditionings, we were unable to obtain any MEPs. Before and after each conditioning, cortical MEPs were measured in the same manner as that described for Experiment 1.

2.5.4. Experiment 4: Duration of QPS aftereffects

Seven subjects participated in this experiment. One subject, who did not participate in Experiment 1, 2, or 3, was enrolled in this experiment. We explored QPS-sub-360 for conditioning in this experiment. Every 15 min for 90 min, 20 cortical MEPs (collected every 14.5–15.5 s) were measured.

2.5.5. Experiment 5: Effects of QPS on brain-stem MEPs

Five subjects who enrolled in Experiment 1 participated in this experiment. We performed brain-stem electrical stimulation to clarify the origins of facilitation after QPS (BST). We explored QPS-supra-360 for conditioning in this experiment.

2.5.5.1. Brain-stem MEPs before and after conditioning. Before QPS-supra-360, eight MEPs to single-pulse BST (brain-stem MEPs) were obtained every 14.5–15.5 s. The stimulation intensity was set to elicit brain-stem MEPs as large as cortical MEPs to a single TMS in resting FDI (about 0.4 mV). The intensity was about 50% of the maximal electrical stimulator output. Then, QPS-supra-360 was applied over the *hot spot* for FDI. After QPS-supra-360, 8 brain-stem MEPs (each brain-stem MEP obtained every 14.5–15.5 s) were collected every 5 min for 30 min.

2.5.5.2. Cortical and brain-stem MEPs obtained in a random order before and after conditioning. Eight cortical MEPs and eight brain-stem MEPs were obtained in a random order for baseline measurement to exclude the effect of prediction of pain associated with BST (i.e., 16 stimuli were given every 14.5–15.5 s in a random order). We measured cortical and brain-stem MEPs in the same manner (random order stimulation) as that in baseline measurement 20 min after QPS-supra-360.

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2.5.6. Experiment 6: Effects of QPS on motor threshold, recruitment curves and somatotopy

2.5.6.1. Motor threshold and recruitment curves. Five subjects who had enrolled in Experiment 1 participated in this experiment. Before and after QPS-supra-360, AMT, RMT and recruitment curves were studied. Stimuli were applied at the optimal site for eliciting cortical MEPs from FDI. After AMT and RMT measurement, eight stimuli were applied at an intensity of 10% below RMT every 7.5–8.5 s. The stimulus intensity was then increased by 5%; another eight stimuli were applied. This process was repeated until the intensity reached 135% RMT. The timing of evaluation after QPS-supra-360 was 20 min post conditioning.

2.5.6.2. Cortical MEPs from the FCU muscle. We recorded cortical MEPs from the right flexor calpi ulnaris muscle (FCU) in these five subjects to confirm topographically specific modulation of the motor cortex after QPS. The optimal site to elicit cortical MEPs from FCU (*hot spot* for FCU, about 1–3 cm medial to the *hot spot* for FDI) was first determined. Then 20 cortical MEPs from the right FCU were obtained by delivering a single-pulse TMS. The stimulus intensity for FCU was set to elicit MEPs with peak-to-peak amplitude of 0.2–0.3 mV. To obtain cortical MEPs from the right FDI, 20 single-pulse TMS were then applied to the *hot spot* for FDI. The QPS-supra-360 was applied over the *hot spot* for FDI, as described previously. At 20 min after QPS-supra-360, 20 cortical MEPs were obtained from both FCU and FDI when TMS was given over the *hot spot* for each muscle.

2.5.7. Experiment 7: Safety study

Three subjects who had participated in Experiment 1 were enrolled in this experiment. Surface electromyograms (EMGs) were recorded from the right FDI, FCU, and biceps brachii (BB) muscles. The EMGs were monitored continuously for the spread of excitation (SE) to proximal muscles and post-TMS EMG activity. According to previous safety studies (Pascual-Leone et al., 1993; Chen et al., 1997c), SE was defined as a progressive increase in four consecutive MEPs of FCU or BB muscle by more than 100% of the baseline (average MEP amplitude of the first 12 MEPs evoked by the first 12 QPS trains) because it was not possible to evoke MEPs from the FDI muscle without inducing small MEPs in FCU or BB muscle in our subjects. In many instances, it was difficult to distinguish post-TMS EMG activity from poor muscle relaxation (Chen et al., 1997c). As a safety precaution, post-TMS EMG activity was defined as isolated EMG activity of shorter than 50 ms and larger than 100 μV in the 1000 ms following a QPS train. We explored QPS-supra-360 or QPS-sham-360 for conditioning to compare the appearance rates of post-TMS EMG activity for these types of conditioning.

2.6. Data analysis and statistics

Data were analyzed using software (SPSS ver. 13.0 for Windows; SPSS Inc.). The effect during conditioning was analyzed using two-way repeated-measures analysis of variance (ANOVA) (between-subject factor, conditioning; within-subject factor, time). The aftereffect of different conditioning was analyzed using two-way repeated ANOVA (between-subject factor, conditioning; within-subject factor, time). For analysis of conditioning parameters, we conducted three-way factorial repeated-measures ANOVA to compare time courses of the four conditioning types (QPS-supra-360, QPS-sub-360, QPS-supra-180, and QPS-sub-180). The intensity (supra-threshold and sub-threshold), the number of trains of QPS (360 and 180 trains), and times of seven levels (baseline, 0–5, 5–10, 10–15, 15–20, 20–25, and 25–30 min) were independent variables. The dependent variable was the normalized amplitude of cortical MEPs. The aftereffect of each conditioning type on MEP sizes was analyzed using one-factor ANOVA (within-subject factor, time).

The effect of the stimulation pattern (single-pulse TMS and brain-stem electrical stimulation) after QPS-supra-360 on MEP sizes was evaluated using two-way repeated-measures ANOVA (within-subject factors: stimulation pattern, time). Recruitment curves before and after QPS-supra-360 were compared using two-way repeated-measures ANOVA (within-subject factors: pre and post QPS-supra-360, and intensity). Paired *t*-tests were used to compare variables (RMT, AMT, and the absolute MEP amplitudes recorded from FCU) before and after QPS-supra-360. The Greenhouse-Geisser correction was used as necessary to correct nonsphericity. Bonferroni's post hoc method was used for further analyses: p values less than 0.05 were considered significant. All figures depict group data.

3. Results

No subject reported any adverse effect during or after any intervention.

3.1. Experiment 1: Comparison between QPS and PPS with the same number of trains

3.1.1. MEPs recorded from FDI muscles during conditioning

Marked MEP facilitation was apparent throughout QPS-supra-360 (Fig. 2a and b). In contrast, PPS-supra-360 showed a slight increase in the MEP amplitude (Fig. 2b) (two-way repeated ANOVA: effect of conditioning, $F[1,22] = 8.415$, $p = 0.008$; effect of time, $F[2.313, 50.892] = 4.179$, $p = 0.012$; conditioning × time interaction, $F[2.305, 50.700] = 2.104$, $p = 0.125$). Post hoc analysis revealed a significant facilitation of MEP to one QPS train at the end of QPS-supra-360, although no significant facilitation of MEP to one PPS train at the end of PPS-supra-360 (Fig. 2b) was found.

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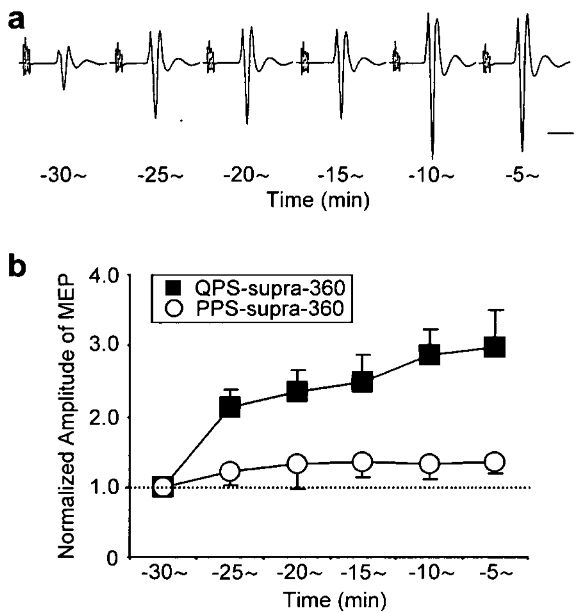

Fig. 2. Comparison between QPS and PPS with the same number of trains: during conditioning. (a) Waveforms of MEPs for 30 min during QPS-supra-360 in a representative subject. Each MEP was elicited by one train of quadro stimuli every 5 s and 360 MEPs were obtained in all. Average waveforms at 5-min intervals were obtained by averaging 60 consecutive responses (started from "−30" min; see Fig. 1). Marked MEP facilitation existed throughout QPS-supra-360. Calibration bars: 20 ms, 0.5 mV. (b) Time courses of normalized MEP amplitudes (mean ± SE) during QPS-supra-360 (filled squares, $n = 12$) and PPS-supra-360 (circles, $n = 12$). During QPS-supra-360 and PPS-supra-360, we obtained a total of 360 MEPs (one MEP elicited by one train every 5 s); the peak-to-peak amplitude of each MEP was measured. A series of 60 MEP amplitudes was averaged to obtain the mean MEP amplitudes at 5 min periods (started from "−30" min, see Fig. 1). Significant effects of conditioning (QPS-supra-360 and PPS-supra-360) ($p < 0.01$) and time ($p < 0.05$) were found, but no significant interaction was found between conditioning and time ($p > 0.05$) using two-way repeated ANOVA, with significant post hoc facilitation of cortical MEPs at the end of QPS-supra-360 (mean ± SE; 281 ± 49%, $t = -2.548$, $p = 0.027$). No significant increase was found in MEPs at the end of PPS-supra-360 (132 ± 15%, $t = -2.047$, $p = 0.062$).

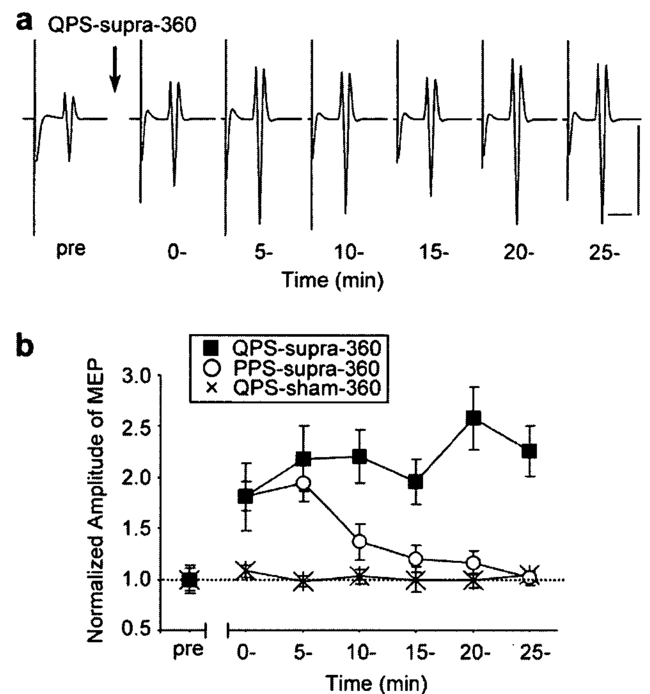

Fig. 3. Comparison between QPS and PPS with the same number of trains: aftereffects. (a) Representative cortical MEPs (i.e., MEPs to single-pulse TMS) before and after QPS-supra-360 from one subject. Averaged waveforms were made from 20 consecutive responses. A marked cortical MEP facilitation was identified after QPS-supra-360 (arrow) for 30 min. Calibration bars: 20 ms, 0.5 mV. (b) Time courses of normalized amplitudes of cortical MEPs (mean ± SE) after three conditioning: QPS-supra-360 (filled squares, $n = 12$), PPS-supra-360 (circles, $n = 12$) and QPS-sham-360 (crosses, $n = 4$). The abscissa shows the time with respect to the conditioning (before conditioning = "pre", immediately after conditioning = "0"). The ordinate shows the normalized amplitude of cortical MEPs. Average amplitudes were calculated from those of 20 consecutive responses obtained every 5 min and normalized to that of baseline measurements. Significant effects of the conditioning ($p < 0.005$) and time ($p < 0.005$) were found; also, a significant interaction between them ($p < 0.001$) was found using two-way repeated ANOVA, with significant post hoc differences between QPS-supra-360 and PPS-supra-360 ($p < 0.05$), and QPS-sham-360 ($p < 0.01$). Cortical MEPs after QPS-supra-360 were facilitated for 30 min after conditioning (post hoc analysis using Bonferroni's method: each time point, $p < 0.05$). After PPS-supra-360, cortical MEPs were facilitated for only 10 min and then reverted to baseline level (post hoc analysis using Bonferroni's method: 0–5 min, $p < 0.05$; 5–10 min, $p < 0.001$; 10–30 min, $p > 0.05$). No significant cortical MEP changes were found after QPS-sham-360 (one-factor ANOVA: effect of time, $p > 0.05$).

3.1.2. MEPs recorded from FDI muscle before and after conditioning

Cortical MEPs were facilitated significantly for 30 min after QPS-supra-360 (Fig. 3a and b), but for only 10 min after PPS-supra-360 (Fig. 3b). No significant cortical MEP changes were found after QPS-sham-360 (Fig. 3b) (two-way repeated ANOVA: effect of conditioning, $F[2,25] = 8.084$, $p = 0.002$; effect of time, $F[6,150] = 4.098$, $p = 0.001$; conditioning × time interaction, $F[12,150] = 4.664$, $p < 0.001$; post hoc analysis: QPS-supra-360 vs. PPS-supra-360, $p = 0.017$; QPS-supra-360 vs. QPS-sham-360, $p = 0.005$).

3.2. Experiment 2: Effect of the intensity and the number of trains of QPS on aftereffects

Three-way repeated ANOVA revealed that QPS aftereffects were affected significantly by the number of trains rather than by the intensity (Fig. 4a) (effect of the number of trains, $F[1,32] = 9.290$, $p = 0.005$; effect of the intensity, $F[1,32] = 0.007$, $p = 0.934$; effect of time, $F[6,192] = 7.585$, $p < 0.001$; the number of trains × time interaction, $F[6,192] = 3.194$, $p = 0.001$; the intensity × time interaction, $F[6,192] = 0.858$, $p = 0.527$; the number of trains × the intensity × time interaction, $F[6,192] = 1.265$, $p = 0.275$). A significant cortical MEP facilitation was found for 30 min, even after QPS-sub-360, but for only 10 min after QPS-supra-180. No significant cortical MEP changes were found after QPS-sub-180 (Fig. 4a).