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Bereitschaftspotential augmentation by neuro-feedback training in Parkinson's disease

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HIGHLIGHTS

- This study showed that Parkinson's disease (PD) patients could restore decreased early component of Bereitschaftspotential (BP) by means of neuro-feedback (NFB) to control negative slow cortical potentials (SCPs).
- It is the first study to demonstrate that decreased pre-movement cortical activity can be restored by endogenous, subject's own effort, without externally driven modulatory stimuli or medication.
- *Good NFB performance of negative SCPs shifts (negativation) most likely increases excitatory field* potentials of pyramidal cells in the supplementary motor area.

ABSTRACT

Objective: Decreased early Bereitschaftspotential (BP) is one of the electrophysiological characteristics in patients with Parkinson's disease (PD). We examined whether PD patients could increase BP amplitude by means of neuro-feedback (NFB) training for their slow cortical potentials (SCPs).
Methods: We worked with 10 PD patients and 11 age-matched controls. BP was measured for self-paced button pressing by their right thumb. The subjects were instructed to make the introspective efforts to produce negative SCPs (negativation). The one-day session consisted of three trials, that is, the first BP, NFB training and the second BP, and each patient performed this routine for 2–4 days. Amplitudes of the first and second BPs were compared between the two groups that were divided depending on NFB performance.
Results: Good NFB performance had the tendency of larger early BP in the second BP recording than in the first one, whereas in the poor NFB performance the early BP was smaller in the second BP recording than in the first one in both patient and normal groups ($p < 0.001$).
Conclusions: Good NFB performance of negativation could increase excitatory field potentials of pyramidal cells for the generation of early BP.
Significance: Voluntary regulation of SCPs could enhance BP in PD patients and in aged controls.

1. Introduction

Parkinson's disease (PD) is a degenerative disorder of the dopaminergic neurons of the central nervous system that impairs motor skills, speech and other functions (Jankovic, 2008). It is characterised by muscle rigidity, tremor, a slowing of physical movements (bradykinesia) and even a loss of physical movements (akinesia) in an extreme situation. The primary motor symptoms are the results of decreased activities of the motor cortices through the basal ganglia–thalamo-cortical loop, caused by the insufficient formation and action of dopamine, which is produced in the dopaminergic neurons of the brain (Zaidel et al., 2009). Anatomical studies of the primate basal ganglia systems show that a major portion of pallidal output is directed to the non-primary motor areas of the frontal cortex, in particular to the supplementary motor area (SMA) (Jurgens, 1984; Schell and Strick, 1984). In humans, it was reported that the SMA is crucial to the organisation of both initial as well as sequential movements (Roland et al., 1980, 1982) and an abnormality of sequencing hand and elbow movements has been demonstrated in patients with unilateral SMA damage (Dick et al., 1986). A similar disturbance of sequenced hand and elbow movements has been demonstrated in patients with PD (Benecke et al., 1987), suggesting impairment of SMA function.

Bereitschaftspotential (BP) is a negative slow electroencephalographic (EEG) potential preceding self-paced movement (Deecke and Kornhuber, 1978). BP consists of an early and a late component (Tamas and Shibasaki, 1985; Shibasaki and Hallett, 2006). The early component of BP could reflect the activity of SMA as its maximum amplitude at the vertex (overlying SMA) regardless of the body parts involved in the movements (i.e., eye, hand, arm or foot) (Deecke and Kornhuber, 1978). The late component is lateralised to the hemisphere contralateral to the side of the movements and is considered to represent activity mainly of the primary motor cortices (Shibasaki et al., 1978; Ikeda et al., 1992).

Dick et al. reported that the early component of BP was smaller in PD patients than in control subjects whereas its late component was not (Dick et al., 1989). Furthermore, the amplitude of the early component of BP was shown to be smaller in the off-phase of L-DOPA medication and returned to normal in the on-phase (Dick et al., 1987).

Scalp-recorded EEG is comprised of a very wide range of oscillatory activities from delta to gamma, and of even direct current (DC) potential shifts, which we call slow cortical potentials (SCPs) in this report. Negative and positive SCP shifts reportedly reflect increase and decrease of the excitability of underlying cortical networks (Birbaumer et al., 1990). In epilepsy patients, negative SCPs are recorded at the time of seizure onset as ictal EEG changes (Ikeda et al., 1996). In recent years, the neuro-feedback (NFB) training was launched as an attempt to reduce seizure frequency by regulating their SCPs presumably as positive as possible in polarity (positivation) (Kotchoubey et al., 1999). In their study the Cz electrode was used to record and control SCPs and the authors showed that cortical excitability could be modified through the effort to control one's own SCPs. Using the NFB system, once they were well trained and became capable of modifying SCPs between the negativity and the positivity shifts, the degree of seizure control became better in the well-trained group than in the poorly trained group (Kotchoubey et al., 2001). We previously reported that, after enough training to regulate their SCPs by means of NFB, young normal subjects produced a larger early component of BP while they were trying to produce negative SCP shifts (negativation), as compared with the BP obtained before NFB training (Fumuro et al., 2010). By contrast, no significant differences were observed in the late component of BP between the two conditions. These results suggested that the self-regulated negative SCPs (negativation) might activate the generators of the early component of BP.

The aim of this study was to clarify whether PD patients could restore their BP amplitude by means of NFB to control negative SCPs (negativation). We therefore, investigated the effects of self-regulation of SCPs on the BP amplitude from 10 PD patients and from 11 age-matched normal controls.

2. Methods

2.1. Subjects

We investigated 10 right-handed patients (two males and eight females) clinically diagnosed as PD aged 36–71 (average of 63) years. Their clinical features are summarised in Table 1. Eleven right-handed normal adults (one male and 10 females) aged 60–69 (average of 65) years were also investigated as controls. The dominant hand was assessed by the Edinburgh Handedness Inventory. In patients with PD, this study was done while medication was continuing (Table 1). All subjects signed the written informed consent form about the study. This study was approved by the Ethics Committee of Kyoto University School of Medicine (No. E-308).

Table 1
Patient profile.

Patient No.	Age (years)/gender	Diagnosis	H-Y grade[a]	LED[b] (mg/d)
1	71/W	PD	III	750
2	71/W	PD	IV	350
3	65/W	PD	III	500
4	69/W	PD	III	600
5	68/W	PD	II–III	325
6	54/M	PD	I–II	350
7	36/M	PD	IV	720
8	71/W	PD	IV	413
9	56/W	PD	I–II	125
10	71/W	PD	II	305

W: woman; M: man; PD: Parkinson's disease.
The actual study was done while the medication was one in each patient.
[a] H–Y grade = Hoehn and Yahr grade (Hoehn and Yahr, 1967).
[b] LED = levodopa equivalent dose calculated by Tomlinson et al. (2010).

2.2. Recording condition

Five Ag/AgCl scalp electrodes placed at C3, C1, Cz, C2 and C4 according to the International 10–10 System were used for EEG recording. An electro-oculogram (EOG) was recorded for horizontal and vertical eye movements by placing the four skin electrodes on the bilateral outer canthi and on the upper and lower edges of the left orbital, respectively. An electromyogram (EMG) was recorded from the right abductor pollicis brevis muscle by a pair of skin electrode over the muscle.

EEGs, EOGs, EMGs and input signals made by pressing the button with the subject's right hand were recorded using a DC-EEG feedback system NEURO PRAX (neuroConn GmbH, Ilmenau, Germany) (Schellhorn et al., 2004).

As the main concern of this study was to record SCPs or DC potentials reliably, we confirmed the following four conditions completely (Fumuro et al., 2010):

(1) NEURO PRAX contains a DC differential amplifier, which has a huge size of input impedance of >10 gigaohm. The huge size of the input impedance minimises the effect of electrode impedance and further enables us to record stable DC potentials without a high-pass filter (Cooper et al., 1980).
(2) Sintered Ag/AgCl electrodes were soaked in a Ten20 Conductive Paste (Weaver and Company, Aurora, CO, USA) for more than 30 min before measurement in order to stabilise the polarisation potential of the electrodes. These electrodes had lower polarisation potentials and were shown to provide stable recording condition (Tallgren et al., 2005).
(3) Before electrode attachment, the skin on which to place the surface electrodes was cleaned by both cleansing paste (Skin Pure; Nihon Kohden, Tokyo, Japan) and sanitary cotton moistened with ethanol and then electrodes as described above were firmly attached.
(4) Before actual recording sessions, we checked and confirmed that the offset voltages of all the electrodes were within ±20 mV range relative to the A1, and the standard deviation of the drifts were <1000 μV. We also visually confirmed that there was no noticeable, even very low, amplitude or very

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Table 2
Analysis of NFB performance and BP (session-based and subject-based).

	PD patients (n = 10)	Healthy volunteers (n = 11)
(1) Session-based analysis		
The total number of one-day sessions	27	31
NFB performance analysis[a]	17	20
Good NFB performance	8 (47%)	12 (60%)
Poor NFB performance	9 (53%)	8 (40%)
BP analysis[b]	13	17
Good NFB performance	7	10
Poor NFB performance	6	7
(2) Subject-based analysis		
Total number of subjects	10	11
The number of subjects for BP analysis[c]	7	9
The number of good NFB group	4	5
The number of poor NFB group	3	4

[a] The first-day sessions (n = 10 and 11) were excluded for further analysis in PD and normals. It was because that in the initial training period subjects could not be familiar with or not good at this internal neuro-feedback process which could distort the finding erroneously.
[b] The first-day sessions (n = 10 and 11) and one-day sessions of poor BP reproducibility (n = 4 and 3) were excluded for further analysis in PD and normals.
[c] Subjects having only single one-day session (n = 1 and 1) and poor BP reproducibility (n = 2 and 1) were excluded from further analysis in PD and normals.

slow artefacts on the EEG waveforms before and throughout the measurement. Ordinary impedance check with 10 Hz alternating current used for conventional EEG, that is, AC potential, recording was not performed.

All signals were digitised at the sampling rate of 128 Hz and band-pass filter from DC to 30 Hz was applied. The subjects sat in a chair comfortably with their arms and hands on the armrests. A computer screen was placed in front of the subjects 130–150 cm from their faces. The eye movement-correction program built into the NEURO PRAX was used to diminish eye movement artefacts. EEG signals were referred to A2 during both NFB training and BP recording.

2.3. Task

2.3.1. Procedural overview

The subjects performed three kinds of tasks in 1 day in the following order: (1) the first BP recording, followed by a 5-min break; (2) NFB training to regulate SCPs (2–5 times of sub-sessions with 3-min breaks), followed by a 5-min break; and (3) the second BP recording while subjects made introspective efforts that produced the negative SCP shifts (negativation) (Fig. 1). The subjects repeated the set of procedures (1)–(3) (1-day session) as described above 2–4 times each on another day, with 1–6 days of interval in between. The BP recording and the NFB training on the first day for each subject were regarded as rehearsal sessions and thus

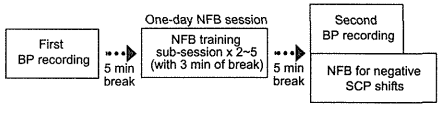

Fig. 1. Procedural overview. Subjects performed 3 kinds of tasks in one day in the following order: (1) the first BP recording, followed by 5 min of break, (2) NFB training to regulate SCPs (2–5 times of sub-sessions with 3 min of break), followed by 5 min of break, (3) the second BP recording while subjects made introspective efforts that produced the negative SCP shifts.

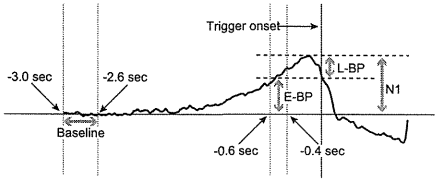

Fig. 2. Schema of BP components and its measurement. Baseline: mean amplitude of the movement-related cortical potentials (MRCPs) between 3.0 and 2.6 s before the trigger onset. Early BP (E-BP): mean amplitude of the MRCPs between 0.6 and 0.4 s before the trigger onset. Pre-movement negative peak (N1): the largest pre-movement negative potentials between −0.5 s and the trigger onset time measured from the baseline. Late-BP (L-BP): this steeper negativity was obtained by subtracting the E-BP from N1.

those were not included for the final analysis. This was not informed to the subjects before examination.

2.3.2. BP recording

The subjects were instructed to look at the blank screen in front of them but not to fixate on a certain point. They were told not to blink from 3 s before to 1 s after the button-press. They performed self-paced, brisk button-press by their right thumb about every 10 s. All subjects had approximately 100 trials of button-press, which was sufficient to obtain approximately 80 epochs of artefact-free trials. EEG signals were referred to as A2 during BP recording, and the reference was changed to linked reference of A1 and A2 for BP analysis.

Four seconds of analysis epochs from −3 to +1 s to the onset of button-press were cut out from the raw data in an off-line manner; EEG data during BP recording were first linearly detrended for each 4 s of the EEG segment. Applied detrend could work for a linear fit (in the least-squares sense) to EEG data and then remove the inappropriately occurring trend from it. The epochs containing artefacts exceeding 150 μV in amplitude (from negative peak to positive peak or vice versa) in the EEG channels were excluded. Artefact-free EEG epochs were precisely aligned to the trigger signal of button-press and averaged. EEG baseline was defined as the mean amplitude of the first 0.4 s of the epoch.

At least 50 artefact-free epochs were averaged. To confirm the reproducibility, the epochs were divided into two groups (odd and even numbers of epochs during BP recording) and were averaged to make two subensembles of waveforms. The sessions where two subensembles of BP were not reproducible were excluded from further analysis.We evaluated the following three measures (Fig. 2):

(1) Early BP (E-BP): Mean amplitude of the movement-related cortical potentials (MRCPs) between 0.6 and 0.4 s before the trigger onset. It corresponds to the E-BP (Shibasaki et al., 1980; Shibasaki and Hallett, 2006).
(2) Pre-movement negative peak (N1): The largest pre-movement negative potentials occurring between −0.5 s and the trigger onset measured from the baseline. As this study employed the button-press for the trigger onset instead of surface EMG onset, the pre-movement potentials often peaked immediately before the button-press trigger onset, and thus its amplitude could contain the whole BP and motor potential in Shibasaki and Hallett (2006). Therefore, we termed it the pre-movement negative peak.
(3) Late-BP (L-BP): This steeper negativity was obtained by subtracting the E-BP from N1 and arbitrarily referred to as L-BP. It mainly corresponds to NS′ (Shibasaki et al., 1980; Shibasaki and Hallett, 2006) although it may partly contain motor potential as explained just above.

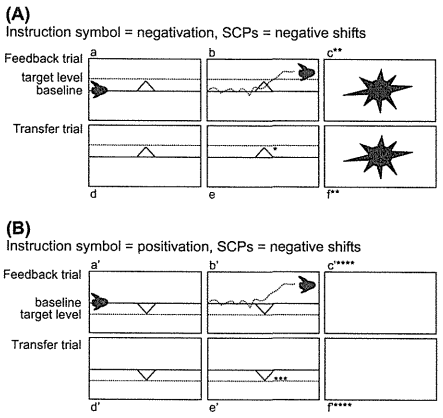

Fig. 3. The actual display during NFB trial. Depending on the task (negative shift or negativation vs. positive shift or positivation), a triangle or reversed triangle appears in the center of the display at the beginning (a, d, a′ and d′). During NFB, the actual state of SCPs is shown as a marker of the fish and it moves toward the right (b, b′). When the subjects successfully control SCPs upon the task, a reward-mark appears in the center at the end (c, f). The same procedures are also done without any appearance of SCP information (without a marker of fish), as shown in d, e, f, d′, e′ and f′. The details are described in the Section 2.3.3. *Instruction symbol to produce negative SCPs shifts (negativation). **The shining sun as the reward mark for successful trial. ***Instruction symbol to produce positive SCPs shifts (positivation). ****Since the SCPs shifts fail to exceed the defined target level, no reward mark appears (Cited and modified from Fumuro et al., 2010).

2.3.3. NFB: self-regulation of SCPs

NFB training to regulate SCPs was done after 5 min of break following the first BP recording. The procedure was the same as the previous study (Kotchoubey et al., 2001; Fumuro et al., 2010).

SCPs recorded from Cz were continuously shown on the front screen as a slowly moving sunfish from the left to the right by 8 s as an almost real-time demonstration (the feedback time delay was around 150 ms) (Fig. 3). The sunfish also shifted upward (negative shift or negativation) or downward (positive shift or positivation), according to the change of SCPs. Namely, the sunfish worked as a visual feedback of the subjects' own SCPs at Cz. Once the sunfish appeared at the left edge, the dichotic instruction symbols appeared in the centre of the screen: the upward or downward arrowhead at the same time (Fig. 3). The direction of the apex of the arrowhead, up or down, indicated the polarity to which the subjects should shift their SCPs.

The subjects were told to regulate the SCPs upon the instruction symbols, and by means of this real-time feedback of SCPs at Cz the subjects tried to learn how to control it by themselves. Therefore, any specific suggestions on how to regulate SCPs were not initially provided by the examiners; the subjects were advised to concentrate on the SCP shift and to find the most successful strategy to move the sunfish towards the required direction. The examiner gave them some examples of mental introspection to control SCPs, which were done and were found useful in the young subjects in our previous study (Fumuro et al., 2010), if they wanted to know.

Each trial lasted 10 s, which was from 2 s before to 8 s after the appearance of the instruction symbols on the centre of the screen. The next trial was separated from the previous trial by random intervals of 1–6 s. Negative- and positive-feedback trials were presented pseudo-randomly at equal probability. NFB training sub-sessions were conducted 2–5 times in 1 day, and each sub-session had 52 trials. The number of sessions was determined based on each subject's condition and fatiguability.

Since this training procedure was expected to be done finally without the apparatus, trials with and without feedback information (the moving sunfish) were intermixed pseudo-randomly in equal numbers, the latter called transfer trials (Fig. 3A and B: lower half).

The trial was judged successful for feedback control once the SCP amplitude exceeded a defined target level and remained at least for 2 s in the last 4 s of the trial. This target level was set by the examiner as follows: in the negative shift trials, the level was set to the range from the baseline to −30 to −50 μV, and in the positive shift trials it was from the baseline to +30 to +50 μV. The EEG baseline was obtained from the first 1 s of the trial. In the case of successful trial, another graphic symbol ('the shining sun') appeared as a reward mark (Fig. 3A: right). The reward marks were also presented in transfer trials when judged successful. The data in the transfer trials were employed for the judgement of performance as described later in the method. The effective visual field for each eye is described below: sunfish: 3 × 3 (degree in horizontal × vertical), arrowhead: 1–2 × 3, the shining sun: 6–8 × 5–7, width of display: 9–10 × 11–13.

After each sub-session, the experimenter asked the subjects their introspective strategies and encouraged them to renew them if the result of the previous sub-session did not have many successful trials.

3. Analysis

3.1. Analysis of NFB (Table 2: upper half)

EEGs of the transfer trials in the latter half of the two to five sub-sessions out of 1-day training were processed by a high-frequency filter of 2 Hz, and then separately averaged for the negativity and positivity trials. (In the case of a total of three or five sub-sessions, the second or third sub-session was included in the average.) Trials containing artefacts exceeding 200 μV (peak to peak) in any of the EEG channels were excluded from the average.

A 1-day session was classified as a good NFB performance if the averaged waveforms of the negativity trials exceeded that of the positivity trials of at least three electrodes including Cz in the period of 2–6 s after the visual symbol appeared on the left (Fig. 4: left). Otherwise, it was classified as a poor NFB performance (Fig. 4: right).

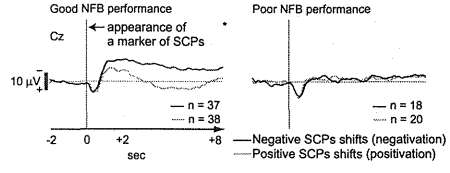

Fig. 4. Example of averaged waveform of the SCPs shift in the good NFB one-day session (left) and poor NFB one-day session (right) (a normal control). The thick line is the results of negative SCPs performance (negativation), and thin lines for the positive SCPs performance (positivation). n = the number of trials. *If averaged waveforms of the negativity trials exceed that of the positivity trials at least 3 electrodes including Cz during this time period, it was judged as good performance. Otherwise, judged as poor.

3.2. Analysis of BP (Table 2: lower half)

All statistical analyses were performed with SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). Analysis was performed for three BP components (E-BP, N1 and L-BP) at Cz because that mean differences between the first and second BP amplitudes were the most prominent at Cz as compared with other electrodes and that the visual feedback of the subjects' own SCPs was recorded from Cz during NFB training. The Shapiro–Wilk test was used to assess for normality; if normality is accepted, analysis of variance (ANOVA) was used. Otherwise, the Mann–Whitney *U* test was used. In order to take the multiplicity of comparisons into account, *p* values were corrected with Holm's correction in all statistical analyses. In order to strengthen the findings, we have analysed all the data in the session-based and subject-based analyses as follows.

3.2.1. Session-based analysis (Figs. 5–7)

The first 1-day sessions in all the subjects and any 1-day sessions without reproducibility of subensembles of BP waveform were excluded, and then the mean difference between the first and second BP amplitudes was compared. In subjects who had both good and poor NFB performance, those 1-day sessions were separately enrolled in both good and poor NFB performance, respectively. Two-way ANOVA having two between-subject factors (1 – disease: PD patients and normal controls, 2 – NFB performance: good and poor NFB performance) was conducted to analyse the mean difference between the first and the second BP amplitudes at Cz.

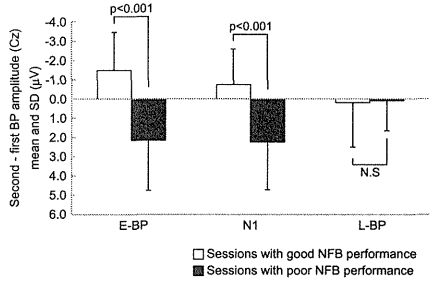

Fig. 5. Effects of NFB performance on BP for Cz. Each bar indicates the mean difference between the second and first BP amplitudes at Cz (the second BP amplitude was subtracted from the first BP amplitude). The second E-BP and N1 tended to be larger than the first ones in the good NFB one-day sessions, whereas in the poor NFB performance the second E-BP and N1 tended to be smaller than the first one. L-BP did not show any difference. In good NFB performance, the number of trials of BP was 82 ± 17 (mean ± SD) in the first BP, and 82 ± 16 in the second BP. Similarly, in poor NFB performance, the number was 97 ± 30 and 92 ± 23, respectively.

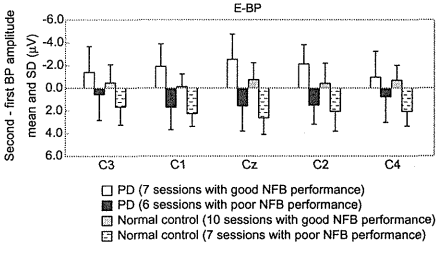

Fig. 6. Effects of NFB performance on early BP for scalp distribution. Each bar indicates the mean difference between the second and first BP amplitudes at each electrode (the second BP amplitude was subtracted from the first BP amplitude). Cz showed the maximum value with bilateral attenuation.

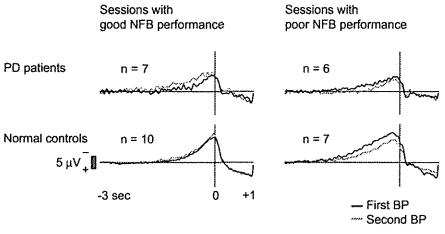

Fig. 7. Grand-averaged BP in PD patients and normal controls (Cz) by session-based analysis. The thick waveform shows the BP measured before the NFB training (first BP). The thin one was the BP (second BP) measured after the training while self-regulated negative SCPs (negativation) was performed. Good NFB performance had the tendency of larger early BP in the second BP than in the first one, whereas the early BP tended to be smaller in the second BP than in the first one in the poor NFB performance in both PD and normal control. *n* = the number of session.

3.2.2. Subject-based analysis

Similar to the session-based analysis, the first 1-day sessions in all the subjects and any 1-day sessions without reproducibility of subensembles of BP waveform were excluded and then the mean difference between the first and second BP amplitudes was compared in each subject. The last 1-day session was adopted for the analysis of the mean difference between the first and second BP amplitudes. All subjects were finally divided into two subject-analysis groups. Two-way ANOVA was conducted to analyse the mean difference between the first and the second BP amplitudes in the same manner as the session-based analysis.

3.2.3. Comparison of BP between PD patients and normal controls

A statistical analysis with the Mann–Whitney *U* test was performed to assess the difference of the first and second BP between PD patients and normal controls at Cz regardless of the degree of their NFB performance. This analysis adopted the data of the session-based BP analysis.

4. Results

4.1. Performance of NFB (Table 2: upper half)

In PD patients, out of a total of 27 sessions of 1-day duration, the first 1-day session in 10 patients was automatically excluded from further analysis. This was because, in the initial training period, these subjects were unable to become familiar with or perform adequately in the internal NFB process, and this could distort the findings erroneously. Eight 1-day sessions were grouped into good NFB performance and nine sessions were into poor NFB performance. In normal controls, out of a total of 31 1-day sessions, the first 1-day session in 11 subjects was excluded automatically from further analysis. Twelve 1-day sessions were grouped into good NFB performance and eight 1-day sessions were into poor NFB performance. To compare the good performance rate between the first 1-day sessions and the second to fourth 1-day sessions, 10 and 11 1-day sessions in the first day were also judged for performance in PD and normals, respectively. The good performance rate was higher in the second to fourth 1-day sessions than in the first 1-day sessions (30% vs. 47% in PD, 36% vs. 60% in normals, though this did not reach statistical significance).

The number of finally accepted 1-day sessions of NFB was similar in both the patient and control groups (17 vs. 20). The number of 1-day sessions with good NFB performance was 47% and 60% in the two groups, respectively.

4.2. Effects of NFB on BP

4.2.1. Session-based analysis

E-BP (Fig. 5, Fig. 6): Two-way ANOVA revealed significant main effect of NFB performance ($F(1,26) = 21.077$, $p < 0.001$). No main effect of disease or interaction attained significance. This means that regardless of PD or normal controls, the second E-BP tended to be larger than the first one in the good NFB 1-day sessions, whereas in the poor NFB performance the second E-BP tended to be smaller than the first one.

N1 (Fig. 5): Two-way ANOVA revealed significant main effect of NFB performance ($F(1,26) = 18.389$, $p < 0.001$). No main effect of disease or interaction attained significance. The result of N1 turned out to have a similar tendency as that of E-BP.

L-BP (Fig. 5): Two-way ANOVA revealed no significant main effect or interaction.

4.2.2. Subject-based analysis (Table 2: lower half)

As the subject-based analysis, four PD patients were grouped into the good NFB group, and three patients were into the poor one. In normal controls, five subjects were placed into the good group and four were into the poor one.

Two-way ANOVA revealed significant main effect of NFB performance on E-BP ($F(1,15) = 12.607$, $p = 0.006$) and N1 ($F(1,15) = 18.800$, $p = 0.002$). No main effect of disease or interaction attained significance. Similar to session-based analysis, this means that regardless of PD or normal controls, the second E-BP and N1 tended to be larger than the first ones in the good NFB group, whereas the poor NFB group tended to have smaller E-BP and N1 in the second BP than in the first one. By contrast, no significant differences were observed in the L-BP (no figures shown).

4.3. Comparison of BP between PD patients and normal controls (Fig. 8

The Mann–Whitney *U* test revealed significant difference of the session-based first BP between PD patients and normal controls in N1 ($p = 0.035$) and E-BP ($p = 0.039$). No significant difference was found in L-BP. This means that the first E-BP and N1 were smaller in PD than those in normal controls, whereas L-BP did not reach the significant difference. The result of the second N1 and E-BP turned out to be a similar significant difference as the first one (N1: $p = 0.021$, E-BP: $p = 0.039$, L-BP: not significant).

5. Discussion

Brain plasticity, brain–computer interface and neuromodulation are the most important concerns in current clinical neuroscience. Deep-brain stimulation in patients with PD provided the great success at least to lessen the patients' symptoms. It belongs to the externally regulated or exogenous procedure of neuromodulation. BP was modulated by TMS previously (Rossi et al., 2000); however, little has been investigated regarding an intrinsic, endogenous procedure for BP modulation.

We previously reported that, after enough training to regulate their SCPs by means of NFB, young normal controls produced a larger early component of BP while they were trying to produce negative SCP shifts (negativation), as compared with BP obtained before NFB training (Fumuro et al., 2010). By contrast, no significant differences were observed in the late component of BP between the two conditions. These results suggested that the self-regulated negative SCPs (negativation) could activate the generators of early component of BP.

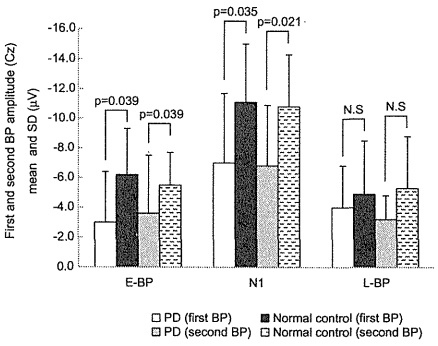

Fig. 8. Comparison of BP at Cz between PD patients and normal controls. Each bar indicates the mean BP amplitude at Cz. PD patients had smaller early BP than aged normal controls, but not for late component of BP.

The aim of this study was to clarify whether PD patients could restore their BP amplitude by means of NFB to control negative SCPs (negativation). This study presented the first scientific evidence that MRCPs could be restored by the subject's own effort, instead of the external stimuli or medication in patients with PD and aged normal controls. As results, with regard to BP, similar to the previous study (Dick et al., 1989), PD patients had smaller E-BP than aged normal controls, but not for the late component of BP (Fig. 8). This suggests that the situation in the present study of MRCP is very consistent with the previous study.

We could summarise the main finding as follows. Good NFB performance had the tendency of larger, E-BP in the second BP than in the first one, whereas the E-BP tended to be smaller in the second BP than in the first one in the poor NFB performance in both PD and normal control (Figs. 5–7).

The present study provides us with the very important concerns as follows: (1) decreased BP in patients with PD could be potentially reversible as long as NFB was well performed or the non-pharmacological process optimally works in the brain and (2) neurophysiological BP modulation is mainly for its early component rather than the late one immediately before voluntary movement onset. With regard to (1), it was previously reported that administration of L-DOPA in normal controls could significantly increase the amplitude of BP (Dick et al., 1987). Namely, not only administration of dopaminergic drugs but also neurophysiological intrinsic activity commonly modify brain activity. Deep-brain stimulation could lessen the clinical symptoms of patients with PD, but it remains to be solved whether the present study or neuromodulation of BP would lessen the symptoms of patients with PD (or not) in future studies. As regards (2) or generators of BP, surface-negative BP is assumed to reflect field potential, probably excitatory postsynaptic potentials (EPSPs) in the superficial layer of the apical dendrite of cortical pyramidal neurons as the result of the thalamo-cortical input (Hashimoto et al., 1979). The current consensus of the generators of the early component of BP is that it begins in the pre-SMA with no site specificity and is followed by the SMA proper according to the somatotopic organisa-

tion, as well as in the lateral motor cortices bilaterally, again with relatively clear somatotopy (Ikeda et al., 1992, 1995; Yazawa et al., 2000).

The late component of BP occurs in the contralateral M1 and the lateral motor cortices with precise somatotopy (Neshige et al., 1988; Shibasaki and Hallett, 2006). With regard to the effect of NFB on the E-BP component, the contribution of electrical fields arising from at least bilateral pre-SMA and SMA proper for the midline component was most likely. From this point of view, SMA activation by means of the self-regulated negative SCPs would act to restore E-BP in PD patients, which showed hypofunction in the SMA as reported before. This implication is consistent with previous study by using functional magnetic resonance imaging (fMRI): a strong positive correlation between negativation of SCP and blood oxygenation level-dependent (BOLD) contrasts was found in the hippocampus and the SMA (Hinterberger et al., 2003). According to these findings, the increased amplitude of E-BP while the negativation task was performed can be interpreted as efficient thalamo-cortical modulation via at least SMA.

With regard to the cortical generators of scalp-recorded SCP, NFB in the present study may share the features of pursuit such as visuo-motor performance using a BP trajectory definitely:

(1) It could affect SCP amplitude because of co-occurrence of pursuit related BP. In addition, NFB in the present study also may share the features of the methods of contingent negative variation (CNV), and at least the SMA is activated during the CNV paradigm. During NFB performance, similar to the trials with visual feedback ('sunfish', in Fig. 3), subjects might have had an image of moving fish in their mind even during the trials without visual feedback.
(2) It could affect the amplitude of the second BP because of co-occurrence of memory-guided pursuit imaging CNV.

For (1), the amplitude of scalp-recorded BP is usually rather small (several μV) as compared with SCP amplitude (10–20 μV); its effect could be practically little. For (2), it is still possible and should be carefully taken into account.

There is a different finding in BP between the present study and our previous one (Fumuro et al., 2010) in the poor-performance situation as follows. In the previous study, young subjects showed increased E-BP amplitude with good NFB performance in the second BP recording as compared with the first recording. In the poor NFB performance, they did not show significant difference of E-BP between the first and second BP recording. In the present study, good NFB performance showed more or less a similar tendency of E-BP as seen in young normal controls. However, the E-BP tended to be smaller in the second BP than that in the first one in the poor NFB performance in both PD and normal controls (Figs. 5–7). This tendency may reflect the effects of probably central fatigue in aged normal controls. In the present study, an entire examination of a single session took 2–3 h, including the time for preparation. The repetitive process of button-press would cause fatigue and result in the decrement of the BP usually (Dirnberger et al., 2004). In the good NFB performance, this decrement may be cancelled out or rather more exceed its former state by the good negative SCP performance. By contrast, fatigue may directly affect BP in the poor NFB performance. However, in the young subjects, the degree of fatigue would be just so small that the degree of NFB performance more directly affected the BP amplitude. Another possible cause for this tendency is the different degree of adaptation to the task. As described in previous literature (Shibasaki and Hallett, 2006), more intention would result in larger amplitude of BP. For instance, in sessions with poor NFB performance, the subjects may have higher motor commands to execute the task in the recording of the first BP; then they may gradually adapt to the optimised commands or efforts to adjust the task, resulting in normal motor control. Although sessions with poor adaptability were excluded from the final analysis, we could not completely exclude this possibility.

There are several limitations in the present study. First, the terms of the NFB training were limited to only 2–4 days. Because of the short term of NFB training, we could not verify whether each subject achieved a better NFB performance day by day or not. Therefore, the grouping by 1-day sessions required further validation as to whether the NFB performance improved much more with lesser artefact. In order to have artefact-free data, as described previously (in the Methods), (1) the notion that averaged waveform of the SCPs should show reproducibility was verified in several electrodes, (2) in BP measurement, epochs containing artefacts were excluded in an objective manner and (3) epochs were separately averaged into two BP waveforms and any measurements with poorly reproducible BP were excluded from further analysis. Through these processes, most artefacts should be excluded from the result and final analysis had the minimised artefact effects. In addition, we compared the two BP waveforms that are measured in the same day, for reducing the effect of various factors such as level of intention and preparatory state that affect the magnitude of BP (Shibasaki and Hallett, 2006). Second, we put together all the PD patients with mild-to-severe degree of Hoehn and Yahr grade (H–Y grade) (Hoehn and Yahr, 1967) into one group. It is possible that each grade has different neurobiological mechanisms and therefore different BP characteristics. Third, the underlying working mechanism of NFB training remains to be solved. It is probably closely associated with the guided activation theory, rather than the reinforcement learning theory although there is some overlap between the two. It is also related to free will but partly to the automatically guided action since the dichotic instruction symbols let subjects decide either direction of NFB procedure.

Good and poor outcome are in almost the same possibilities not only in the present but also previous study (Kotchoubey et al., 1999). About 50% of the good outcome may be criticised as it is by chance or chance-level. However, this is unlikely because of the following two reasons: (1) when we compared good outcome rate between the first 1-day sessions and the second to fourth 1-day sessions, it was higher in the second to fourth 1-day sessions than in the first 1-day sessions (30% vs. 47% in PD, 36% vs. 60% in normal controls) (although it did not reach statistical significance) and (2) Kotchoubey et al. (1997) showed that many training sessions produced higher rate of good outcome; namely, after 35 training sessions, 13 among 18 patients (=72%) showed so-called good training performance. This suggests that longer training is needed to obtain a higher rate of good training outcome.

As a better successful rate in NFB training is essential for clinical applications, a more effective training method should be developed. Good regulation of the SCP could provide us with better clinical application. Therefore, it is also very important to delineate any factors to enhance or predict good training performance for clinical application. For example, since event-related synchronisation or desynchronisation of background EEG could reflect the intercortical or cortico-subcortical network interaction, a further analysis with SCP may provide us with any predictable information in this regard in the future. Furthermore, future studies could clarify the effect of NFB on BP in PD patients with different degree of symptoms.

6. Conclusions

Good NFB performance of negative shifts (negativation) could increase excitatory field potential, probably EPSP of pyramidal cells for the early component of BP generation in PD patients and aged normal controls. NFB could enhance the excitability of cortices related to voluntary movement preparation, and a further investigation will be warranted for improvement of clinical motor function of PD in relation to BP restoration.

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RESEARCH ARTICLE

Mirror visual feedback can induce motor learning in patients with callosal disconnection

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Abstract Mirror therapy using mirror visual feedback (MVF) has been applied to the stroke rehabilitation of hemiparesis. One possible mechanism of mirror therapy is the functional interhemispheric connectivity between sensorimotor areas via corpus callosum. To test this hypothesis, we investigated the MVF-induced motor learning in 2 patients with callosal disconnection. Callosal connection in patients was evaluated by clinical measures and the interhemispheric inhibition (IHI) using transcranial magnetic stimulation. Both patients suffered from somatosensory cognitive disconnection, and one showed the loss of IHI. Motor training with MVF significantly improved the motor behavior of both patients. Extending our previous study, the results of callosal patients suggested that the visual feedback through a mirror might play the crucial important role for the improvement of motor performance, rather than interhemispheric interaction via corpus callosum.

Keywords Mirror visual feedback · Transcranial magnetic stimulation · Motor learning · Corpus callosum

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Introduction

Mirror therapy is a new rehabilitation technique, which was originally introduced to alleviate phantom limb pain in amputees (Ramachandran and Rogers-Ramachandran 1996). By superimposing the visual image of the intact limb on the phantom one using a mirror visual feedback (MVF), patients are relieved from the chronic pain of their amputated arm. Following this study, successful use of mirror therapy has been reported in patients with other pain syndromes (Moseley 2004; Rosen and Lundborg 2005). Furthermore, studies in stroke suggested that mirror therapy may be beneficial for motor recovery of the paretic arm (Altschuler et al. 1999; Sathian et al. 2000; Stevens and Stoykov 2003; Sutbeyaz et al. 2007; Yavuzer et al. 2008). Altschuler et al. reported that MVF can improve the motor performance of chronic stroke patients using a crossover design (Altschuler et al. 1999). Subsequently, randomized controlled trials have also reported the clinical efficacy of MVF in patients with subacute (Yavuzer et al. 2008) and acute stroke (Dohle et al. 2009).

Although the clinical application of MVF is widely noticed in the field of neurorehabilitation, its physiologic mechanism is not fully clarified. Our previous study (Nojima et al. 2012) using transcranial magnetic stimulation (TMS) suggested the importance of visual feedback, but not of the intermanual transfer or interhemispheric interaction for MVF-induced motor learning. Recent studies also suggested that the interhemispheric interaction between both M1 may not play a crucial role for MVF-induced motor learning (Hamzei et al. 2012; Lappchen et al. 2012). However, the neurophysiologic studies in normal volunteers are not sufficient to fully determine the functional role of interhemispheric connectivity in MVF-induced motor learning.

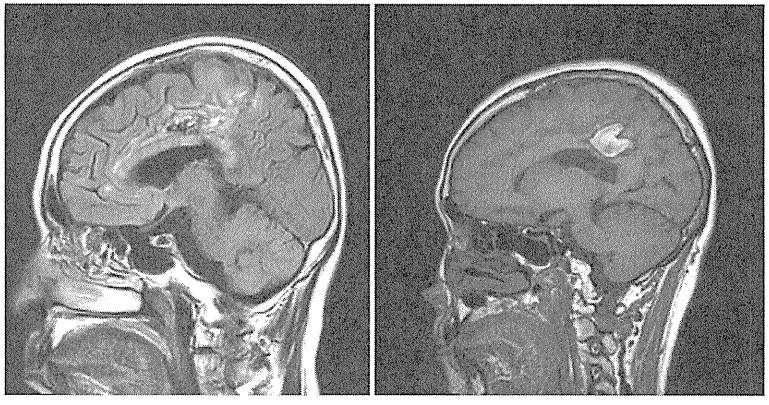

Fig. 1 Magnetic resonance images (FLAIR for Patient 1 and T1 for Patient 2) of 2 patients

To test this point directly, we investigated the MVF-induced motor learning paradigm similar to mirror therapy (Nojima et al. 2012) in 2 patients with corpus callosum lesion. We expected that the MVF-induced motor learning will be lost if the interhemispheric connection between sensorimotor areas is essential for the mirror therapy.

Subjects and methods

Subjects

Two patients with corpus callosum lesion and six age-matched healthy volunteers participated in this study. The study protocol 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 subjects. All subjects gave written informed consents prior to the participation.

Patient 1 is a 69-year-old female suffering from cerebral infarction (Fig. 1a), whose initial symptoms were right-sided mild motor weakness and ideomotor apraxia on her left hand. Patient 2 is a 63-year-old female suffering from cerebral hemorrhage (Fig. 1b), whose initial symptom was the gait disturbance. At the time of testing, they were well recovered and in the chronic stage with almost no residual motor weakness (18 and 6 months after the onset for Patients 1 and 2, respectively).

However, these two patients showed impairment in a test of cross-localization of finger tips (CLF), which constitute the hallmark of the interhemispheric disconnection syndrome (Geffen et al. 1985; Satomi et al. 1991). A CLF test is usually performed as follows: the patients with their eyes closed, hands extended, and palms up is touched on a finger of one hand and asked to touch the corresponding finger of the other hand with the thumb of that hand. CLF disturbance, that is, the inability to perform this task in both directions (right-to-left and vice versa), has been reported in patients with callosal lesions (Volpe et al. 1982; Leiguarda et al. 1989). The fact that the disorder is bidirectional that the localization is normal when carried out with the same hand that is stimulated and that the test is nonverbal suggests that the disturbance is due to callosal disconnection between the somesthetic centers of the two hemispheres.

Six age-matched healthy volunteers (one male and five female) aged 56–78 years (mean ± SD, 71.2 ± 8.2 years) served as controls. None of the subjects had a history of neurological illness, and all were right-handed according to the Edinburgh handedness inventory (Oldfield 1971).

Interhemispheric inhibition (IHI)

To assess the interhemispheric connectivity between the primary motor cortex (M1) of 2 patients, we measured the interhemispheric inhibition (IHI) of the motor-evoked potentials (MEP) induced by TMS. Patients were seated comfortably on armchair with their arms placed on the armrest with the hands facing upward. Surface electromyogram (EMG) was recorded from the first dorsal interosseous (FDI), using pairs of silver electrodes. The recorded EMG

signal was amplified, bandpass filtered (5–2,000 Hz), and digitized at a rate of 10 kHz. Patients were instructed to keep relaxation throughout the experiments with the aid of visual feedback from the EMG monitor.

TMS was given using a figure-of-eight coil (9 cm for the outer diameter) connected to two Magstim 200 stimulators (Magstim Company, Whitland, Dyfed, UK). The coils were placed over the optimal position in the right and left M1 to elicit the best motor response in both FDI muscles with the coil held approximately 45° to the midsagittal line. The optimal position was marked on the scalp by a soft tip pen to ensure identical placement of the coil throughout the experiment. The direction of the induced current was from posterior to anterior.

The method used to measure IHI was similar to the one that reported previously (Ferbert et al. 1992; Koganemaru et al. 2009). The intensity of condition stimulus and the test stimulus was adjusted to produce an MEP of ~1 mV peak-to-peak amplitude in each target muscle. IHI was measured with an interstimulus interval of 10 ms. IHI was expressed as the ratio between the mean peak-to-peak MEP amplitudes in conditioned versus unconditioned trails.

MVF-induced motor learning

Subjects were seated in a comfortable armchair in a totally relaxed position. The target task that was measured before and after MVF training was to rotate two cork balls (diameter 30 mm; weight 5 g) as fast as possible in a counterclockwise direction with their left hand. The dexterity of the motor performance of the left hand was examined by counting the number of two-ball rotations during 30 s. After the experiments, one of the experimenters, who was blind to the participants' status, reviewed the video recording and counted the number of rotations.

The motor learning was accomplished by the mirror visual feedback of the right hand motor training. Patients placed both hands inside a box made of wood and mirrored glass for mirror therapy, and they could not see the right hand directly. The position of the angle of the mirror was carefully adjusted so that the mirror image of the right hand was shown over the real left hand (Fig. 2). They practiced 10 sets of the clockwise rotation of two balls for 30 s each using the right hand with MVF superimposed on the left hand. They were asked to relax their left hand, which was continuously monitored and confirmed by surface EMG. They practiced by completing 10 sets of two-ball rotations for 30 s with a 30-s rest interval to avoid fatigue (total of 270 s).

To assess the intertrial variability and repetition-induced improvement of the target task performance done by the left hand before and after the MVF intervention, we investigated the effect of the right hand training without a mirror on the left hand performance in healthy control. Subjects placed both hands inside a box made of wood and transparent glass. They performed the same motor training of the right hand as that performed by patients without mirror. During the intervention, they were asked to keep looking at the unmoving left hand through transparent glass.

Fig. 2 A mirror box used in the present study. Patients were asked to carefully watch its reflection of right hand movement from the mirror. Their left hand was hidden behind the mirror

For both patients and controls, the number of rotations for the target task (left hand) was measured before and immediately after the intervention.

To measure the motor learning effect after MVF intervention, the ratio of the number of ball rotation was calculated. The cutoff value for the increased motor performance was set at the 2 standard deviation above the mean ratio in healthy controls without MVF.

Results

For Patient 1, the IHI from the right to the left M1 and vice versa were 1.11 and 1.01, respectively. For Patient 2, those were 0.42 and 0.70. The numbers of rotations for the target task before and after intervention in Patient 1 were 13 and 16, respectively. In Patient 2, those were 8 and 12, respectively. For the MVF-induced motor learning, therefore, both Patients 1 and 2 showed the increase in motor performance, 123 and 150 % in the ratio of number of ball rotations.

On the other hand, the control experiment without a mirror in healthy age-matched controls showed 14.3 ± 5.7 and 14.2 ± 7.1 rotations before and after the intervention and 96 ± 13 % in the ratio of number of ball rotations. Although the baseline motor performance (the number of rotations before training) was significantly poorer in healthy aged

volunteers ($p < 0.05$) compared to the young volunteers (20.4 ± 3.8, Nojima et al. 2012), the motor performance of patients was similar to that of the age-matched control group.

The effects of MVF were above the cutoff threshold (122 %) for both patients. The mean effect size of MVF-induced motor learning in patients was similar to our previous study (136.5 %) (Nojima et al. 2012).

Discussion

In this study, we found a significant improvement of motor performance by intervention with MVF in two patients with callosal lesion. Clinical evaluation indicated the interhemispheric disconnection of the somatosensory function in Patients 1 and 2. The measurement of IHI using TMS suggested the loss of connectivity between bilateral M1 in Patient 1. Thus, it is likely that the interhemispheric connection is not essential for the motor learning associated with MVF.

This result is in accord with our previous study (Nojima et al. 2012) in healthy subjects that repetitive motor training of the nontarget hand significantly improved the motor performance of the target hand if MVF was given. Recent studies in normal volunteers also suggested that the interhemispheric interaction between both M1 does not play a crucial role in mirror therapy. By using fMRI, Hamzei et al. found that mirror therapy enhanced the various interregional functional interactions, but not that between bilateral M1 (Hamzei et al. 2012). Moreover, recent TMS study reported that the IHI before and after mirror training was not significantly different (Lappchen et al. 2012). In addition, previous studies (Garry et al. 2005; Shinoura et al. 2008) showed that the excitability in motor-related areas contralateral to the target hand was facilitated by training with MVF as well as our previous study (Nojima et al. 2012).

It has been reported that complete section of the corpus callosum abolishes or greatly reduces the ability to transfer and integrate information between the cerebral hemispheres (Sauerwein and Lassonde 1997). The callosal disconnection can be demonstrated when input is restricted to a single hemisphere and the other. We confirmed the callosal disconnection of patients using clinical evaluation, MRI and IHI measurement. The CLF task, which required transfer of a tactile stimulation between hands, is sensitive to disconnection deficits (Geffen et al. 1985) and is often performed at the bedside to assess the extent of deficit and to follow the path of recovery. Lesions in corpus callosum area are clearly depicted in MRI scan of both patients. In addition, IHI evaluation gives crucial information regarding the function of bihemispheric connection of M1. Taken together, Patient 1 showed mostly complete sensorimotor callosal disconnection, whereas Patient 2 was partly. Some previous reports have demonstrated that deficits in interhemispheric motor integration were associated with lesions of the anterior third of the corpus callosum (Jeeves et al. 1979; Jeeves and Silver 1988), whereas somesthetic transfer has suggested to be impaired owing to the lesion of the body of the corpus callosum (Bentin et al. 1984; Geffen et al. 1985; Leiguarda et al. 1989).

With regard to the connectivity of bilateral M1, it reported functional connection with transcallosal fiber influenced on bilateral motor and cognitive function (Nishikawa et al. 2001; Tomaiuolo et al. 2001). When bilateral TMS was given to each hemisphere, the conditioning effect of the other M1 stimulation can be found as short as from 6 to 10 ms (Ferbert et al. 1992; Gerloff et al. 1998). One of the fastest connectivities between bilateral M1 is transcallosal fibers, whose corticocortical conduction time should be around 7–15 ms measured in myoclonic patients (Shibasaki et al. 1978; Brown et al. 1991; Gerloff et al. 1998). The interhemispheric interaction between bilateral M1 is strong and effective via commissural fibers in corpus callosum.

It is probable that the corticospinal system for the visual motor processing might be associated with mirror neuron systems (MNS) (Rosen and Lundborg 2005; Sutbeyaz et al. 2007). Several functional brain imaging studies investigated human MNS and showed that the observation of an action recruits a consistent network of cortical areas (Buccino et al. 2001; Rizzolatti and Craighero 2004; Lui et al. 2008). In one fMRI study (Matthys et al. 2009), the superior temporal gyrus was activated during MVF intervention, suggesting a link between MVF and MNS. Therefore, one possible mechanism for MVF-induced motor learning may be MNS-like properties of M1.

The present study in patients suggests that motor training with MVF can induce the improvement of motor performance without callosal connection. The MVF might facilitate the excitability of M1 by activation of the motor-related cortical network, such as MNS.

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Spike-timing dependent plasticity(STDP)

——ヒトでの連合性対刺激による可塑性を中心に

Spike-timing dependent plasticity(STDP)
——Topics of human neuroplasticity induced by paired associative stimulation

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◎2つのシナプス結合した神経細胞が時間的に近接して発火することが繰り返されると，シナプス伝達効率が増強される現象はHebbの可塑性と呼ばれる．とくにその場合，2つの発火の時間的関係によって可塑性の極性が長期増強や長期抑制に調節されることが多い(spike-timing dependent plasticity：STDP)．2000年に経頭蓋的磁気刺激法(TMS)と末梢神経電気刺激をペアにして組み合わせる連合性対刺激(PAS)を反復することで，ヒト一次運動野に非侵襲的に可塑性を誘導する手法が開発されて以来，PASによる神経科学研究やその臨床応用はトピックとなっている．本稿ではPASの原理を概説し，末梢神経電気刺激以外を用いたPASの変法や臨床応用について述べる．

Key word：STDP，連合性対刺激(PAS)，可塑性，経頭蓋的磁気刺激法(TMS)

経頭蓋的磁気刺激法(transcranial magnetic stimulation：TMS)は非侵襲的にヒト脳を電気刺激する手法である．単発ではなく，連続刺激(repetitive TMS：rTMS)を可能とする機器が開発されて以降，ヒト脳に人工的に可塑性(plasticity)を引き起こす手法として，リハビリなどの臨床応用はもちろんvirtual lesion studyとして認知神経科学の重要なツールとしても用いられている．

神経可塑性は中枢神経系での持続する機能的・構造的な変化を意味しており，大脳皮質領域マップからNMDA受容体まで種々のレベルで生じる多彩な現象である．ただし，TMSによってヒト脳に誘導される可塑性の場合にはおもにシナプス可塑性，すなわちシナプス伝達の効率の変化を意味することが多い．Hebbが1949年に唱えた神経細胞間情報伝達の可塑的変化が記憶の神経生理学的基盤であるという仮説は，以後の神経科学研究に大きな影響を与えた[1]．Hebbの仮説とは以下のとおりである．

「細胞Aの軸索が細胞Bの興奮を引き起こすのに十分なほど近接して存在し，その発火活動に，反復してまたは持続して関与する場合には，一方の，あるいは双方向の細胞に何らかの成長過程や代謝的な変化が生じ，細胞Bを発火させる細胞群のひとつとして細胞Aの効率が増大する．」

可塑性については，そのシナプス効率が上昇するもの(long-term potentiation：LTP)と，低下するもの(long-term depression：LTD)の2種類がある．rTMSの介入後では5 Hz以上の高頻度刺激では促通，1 Hzの低頻度刺激では抑制が生じるのがその例である．

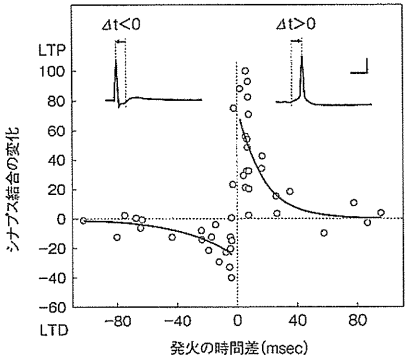

図1 Spike-timing dependent plasticity(STDP)[3]
海馬細胞でのシナプス前神経細胞の発火(シナプス後神経細胞のEPSPの立ち上り)とシナプス後神経細胞の発火のタイミングの時間差によって，LTP/LTDが調節される．

Spike-timing dependent plasticity(STDP)(図1)

しかし，Hebbの定式ともっともよく合致するプロトコールは，連合性対刺激(paired associative stimulation：PAS)による可塑性である．これはあるシナプスにおいて，そのシナプス伝達と(他の入力による)シナプス後神経細胞の脱分極が同時か一定の時間差をもって低頻度で繰り返されることによって，シナプス可塑性が生じる現象を指している．たとえば，嗅内皮質から歯状回への弱い入力と強い入力の2種類の存在する実験系[2]では，弱い入力と同時か数十msec遅れて強い入力があるPASが繰り返された場合には，弱い入力に対してLTPが生じる．一方，強い入力に遅れて弱い入力があるPASを反復すると，弱い入力に対するLTDが生じる．このように2種類の入力のタイミングによってLTPとLTDが生じる現象はspike-timing dependent plasticity(STDP)やHebbian plasticityと呼ばれる．このSTDPは神経細胞[3]，スライス[4,5]，*in vivo*での動物実験[6]で観察される．これらのSTDPのメカニズムには，NMDA受容体とシナプス後神経細胞でのCaレベル上昇の関与が示唆されている(詳細は文献[7]参照)．そして，2つの入力の時間差については，動物種や脳部位によって差はあるものの，おおむね20 msec以内(ときに100 msec)とされる[8]．

ヒト脳でのPASによるSTDP

Stefanらは，TMSと末梢神経電気刺激を組み合わせるPASによってヒト一次運動野(M1)のLTPを誘導する手法を2000年に報告した[9]．具体的には，正中神経手首部の電気刺激の25 msec後に反対側M1の手領域へのTMSを行うPASを，20秒ごと(0.05 Hz)に90ペア施行する(30分)プロトコールで，同部位での運動誘発電位(motor evoked potential：MEP)振幅を50〜70%増大させることができたという内容であった(図2)．この現象は30分以上持続し(24時間後には影響は消失)，体部位特異的(正中神経支配領域)に誘導され，脊髄前角の運動神経細胞の興奮性(F波で測定)は変化させないことから，ヒトM1でのLTPと考えられている．ただし，このPASでH波は増大することが報告されており[10]，MEP振幅の増大に脊髄運動神経細胞の興奮性の変化が関与する可能性はある．

M1へのTMSは経シナプス的に錐体細胞を興奮させるため，このプロトコールでは末梢体性感覚刺激のM1錐体細胞への入力(刺激後20〜25 msec)とほぼ一致かやや遅れてTMSによる経シナプス的な入力がM1錐体細胞に生じる．さらに，このPASによって生じる可塑性は電気刺激の10 msec後にTMSを施行するプロトコール(M1へのTMS後に体性感覚入力が到達する)に変更すると約30%のMEP振幅低下を生み出す[11]．したがって，このPASによるヒトM1可塑性は2つのM1入力の前後関係によってLTP/LTDを生じるという意味で，STDPの一種である．

LTPを誘導するPASでのペア刺激は5〜20秒間隔で提示されることが多い．だが介入時間を短縮するために0.2秒ごと(5 Hz)でのPASも考案されている(rapid PAS)[12]．

PASでの可塑性の生理機構

PASによって生じるヒトM1での可塑性と動物実験でのシナプス可塑性は，同一の生理機構に由来しているのであろうか．いくつかの薬理学的実験結果はこの仮説を支持している．たとえば，

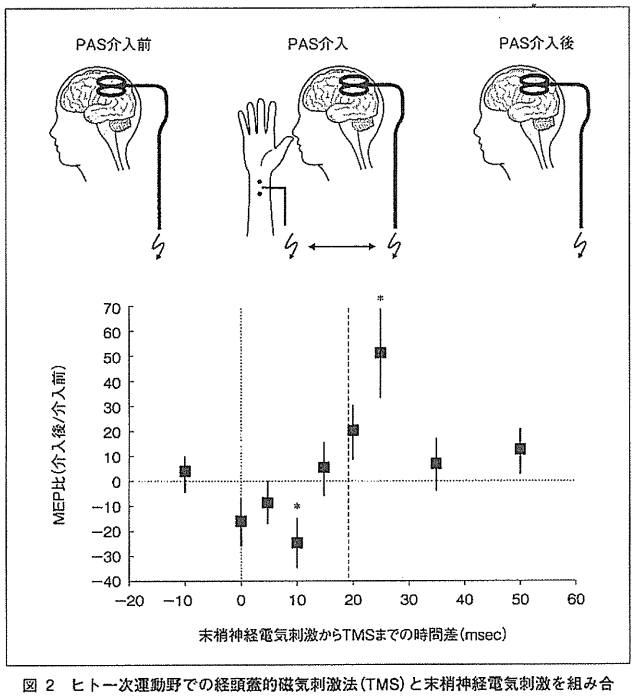

図 2　ヒト一次運動野での経頭蓋的磁気刺激法(TMS)と末梢神経電気刺激を組み合わせた連合性対刺激(PAS)[11]
PAS の時間差が 25 msec では LTP，10 msec では LTD を引き起こしている．
*：$p<0.05$.

NMDA 受容体のアンタゴニスト[13]やL型 voltage-gated Ca チャネルのアンタゴニスト[11]を服用すると，PAS による可塑性の誘導は阻害される．また，BDNF 遺伝子多型と PAS による可塑性の誘導の起こりやすさに関連があるという報告もある[14]．

また，PAS による M1 可塑性と実際の反復運動によって生じる使用依存的可塑性の間には相互作用がある[15,16]．この結果は，日常生活での運動学習や患者での継続的なリハビリの治療効果と PAS によって誘導される人為的な可塑性が，共通の脳内機構をもつことを示している．

PAS による可塑性の誘導の起こりやすさには個人差が大きい[17]．実験を行う場合には，注意[18]，年齢(高齢ほど可塑性が誘導されにくい)[19]などの要素を統制することが必須である．また，運動をよく行う被験者[20]や音楽家[21]では PAS による可塑性が増強しているという報告もある．

そのほかのPASプロトコール

Hebb の仮説に従えば，時間的に近接した2つの入力の繰返しとしての PAS はシナプス可塑性の一般原理である．したがって，末梢神経電気刺激と TMS の組合せ以外にもさまざまなプロトコールが報告されている．たとえば，末梢神経電気刺激と TMS の組合せを一次体性感覚野(S1)に適用し，体性感覚誘発電位の振幅で S1 興奮性を評価すると，末梢神経刺激の 15〜20 msec 後に TMS を与えると LTP，20 msec 前に TMS を与えると LTD が生じる[22]．

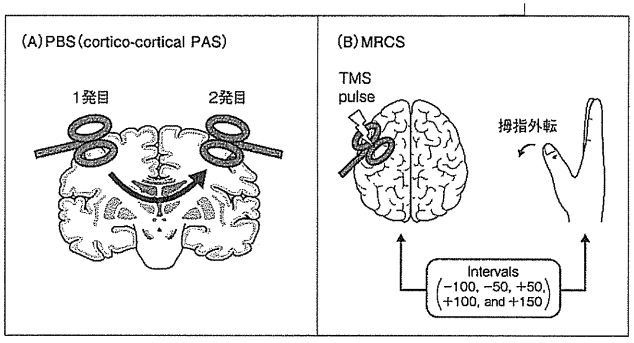

図 3　著者らの考案したPASプロトコール
A：PBS．両側 M1 を 7〜15 msec の時間差で TMS 刺激するペアを繰り返すことで，遅れて刺激されたほう(2発目)の M1 で LTP が誘導される[23]．
B：MRCS．自発運動と M1 への TMS 刺激を組み合わせるペアを繰り返すことで，−50 msec(TMS が運動に先行)では LTP，100 msec(運動後に TMS)では LTD が誘発される[27]．

著者らの考案した新しい PAS のプロトコールとしては paired bihemispheric stimulation(PBS；cortico-cortical PAS)や movement-related cortical stimulation(MRCS)がある(図3)．PBS は，両側 M1 に 7〜15 msec の時間差で TMS を与えるプロトコールである[23,24]．標的 M1 への TMS と反対側 M1 への TMS で生じる脳梁を介して，標的 M1 への皮質皮質間入力とを時間的にほぼ一致させる対刺激を繰り返すことで LTP を誘導する手法である．2カ所の TMS による対刺激を用いる手法としては，腹側運動前野と M1[25]，小脳と M1[26]を組み合わせた PAS が報告されている．MRCS は，視覚刺激に対して運動させる反応時間課題を行うと同時に，その運動開始と一定の時間間隔で運動反対側 M1 に TMS を与える手法である[27]．運動準備や遂行に伴う M1 への皮質皮質間あるいは小脳一視床-皮質の投射入力と TMS を組み合わせるプロトコールである．STDP を示し，運動開始 50 msec 前に TMS を与えると LTP，100 msec 後では LTD を誘導することができる．

臨床応用の可能性

PAS によって誘導される LTP は薬物治療を受けていないパーキンソン病患者では低下または消失し，ドパミン治療によって LTP が回復する[28]．ドパミン系と関連する疾患であるレストレスレッグ症候群[29]や統合失調症[30]でも同様の PAS による可塑性低下が報告されている．一方，ジストニーでは PAS による可塑性や LTP の増強が報告されている[31,32]．また，脳卒中後の麻痺患者へのリハビリ目的での臨床応用も行われつつある[33,34]．

PAS による可塑性は，シナプスのレベルで起きる可塑性とよく似た特性をもつ可塑性をヒトで非侵襲的に誘導することができる興味深い介入プロトコールである[35]．また，対刺激の時間的順序を操作して LTP と LTD を制御できること(STDP)，運動学習や記憶の生理機構と関連していることなどの特性からも，今後，神経科学的な知見の積み重ねと臨床応用への展開が期待される．

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特集「脳機能計測法を基礎から学ぶ人のために」磁気刺激法

脳機能可塑性

野嶌一平[1)]　美馬達哉[2)]

要旨　経頭蓋磁気刺激（transcranical magnetic stimulation: TMS）は，麦動磁場により非侵襲的に大脳皮質を刺激する方法であり，大脳皮質の興奮性，脳損傷後の神経再構築，脳の可塑性発現を知るための有力な手段となっている。これまでの脳機能評価で使用されてきたTMSは単発刺激による評価であったが，連続刺激（repetitive TMS: rTMS）が可能となる機器が開発されて以降，ヒト大脳皮質に対して人工的に可塑的変化（Plasticity）を引き起こす手法として，神経内科領域やリハビリテーション領域などで積極的に臨床応用されている。また近年，より効果的な刺激方法を開発するための様々な試みが行われている。本稿ではTMS，特にrTMSによる大脳皮質の可塑性を誘発する方法とその神経生理学機序に関する知見を紹介する。

はじめに

大脳皮質の電磁気的活動の計測による脳波や脳磁図，脳機能イメージングによる機能的MRIなど従来の脳機能研究手法は，ある特定の課題遂行に関連した脳活動を画像化したり定量化したりするうえで極めて有用である。しかしその脳活動がどのような生理学的意味を持っているかという点については解明に限界があった。一方，経頭蓋磁気刺激（Transcranical Magnetic Stimulation: TMS）を用いた検討では，脳への入力（誘導電流）と脳からの出力（運動誘発電位：Motor Evoked Potential, MEP）を厳密にコントロールして評価することで，脳活動を詳細に解明することが可能となる。MEPを誘発する最低出力である運動閾値，MEPまでの潜時と振幅などの基本的計測以外に，筋肉を随意収縮させている時にTMSを与えることで誘発されるSilent Periodなどを計測することで抑制性の活動評価も可能である。また，磁気刺激装置を2台連結して2連発磁気刺激法を施行した場合，大脳皮質内の抑制機構の定量的評価として用いることもできる。

一方，上述のTMS刺激は単発刺激による評価であったが，反復した刺激（repetitive TMS: rTMS）を行うことで，大脳皮質を中心に人工的に可塑的変化（Plasticity）を誘発できることが報告されて以降，rTMSは臨床に広く応用されている。本稿は脳機能可塑性というテーマであり，特にrTMSによって大脳皮質に可塑的変化を誘発する方法とその機序に関する知見を紹介する。

刺激方法

1. 反復経頭蓋磁気刺激（rTMS）

rTMSは中枢神経機能を可塑的に変化させることが知られており，健常者における脳機能局在の研究や，様々な精神・神経障害に対して治療的に試みられている。本法における特徴として，1 Hz以下の低頻度rTMSは刺激部位に抑制性に作用し，5 Hz以上の高頻度rTMSは興奮性に作用するという刺激頻度に依存した効果が見られることである。一方，rTMSによる大脳皮質の興奮性または抑制性効果の神経生理学的機序についてはまだ不明な点が多いが，長期抑制（Long-Term Depression: LTD）様効果に関係している可能性が報告されている。rTMSが刺激頻度に依存した効果を発現するという報告は1997年にChenら[1)]によって最初に行われた。その研究では，0.9 Hzで115%安静時運動域値という弱い刺激強度で一次運動野（M1）を900回刺激したところ，刺激領域のMEPの振幅が減少し，M1の興奮性が低下することが報告された。一方，5 Hz以上の高頻度rTMSは，低頻度刺激とは反対にM1興奮性を増加させるとされている[2,3)]。

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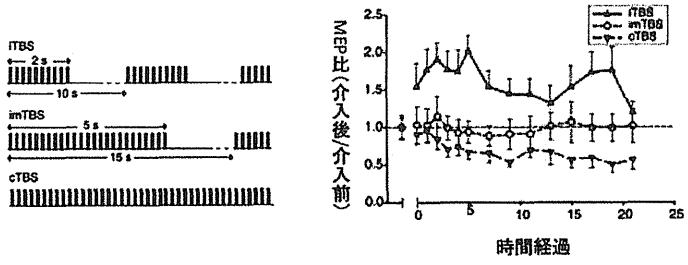

図1　TBS（Theta Burst Stimulation）
3連発刺激を50 Hzの周期（20 msの時間間隔）で行い，これを200 ms間隔で繰り返す，すなわち5 Hz（シータ周波数）の刺激を繰り返す方法である。iTBS（intermittent TBS）では2秒刺激，8秒休止という周期で刺激することでM1の興奮性を増大させることができる。imTBS（intermediate TBS）は5秒バースト刺激，10秒休止という刺激で，これはM1への影響は出現しない。更にcTBS（continuous TBS）ではバースト刺激を連続する方法でM1の抑制を引き起こす。

2. Theta Burst Stimulation（TBS）（図1）

3連発刺激を50 Hzの周期（時間間隔：20 ms）に200 ms間隔で繰り返すことで，5 Hz（シータ周波数）の刺激を与える方法である。刺激方法により興奮性も抑制性も引き起こすことが可能であり，intermittent TBSとcontinuous TBSの2種類の刺激方法が提案されている。前者は興奮性刺激として，一方後者は抑制性刺激として作用することが報告されている[4,5)]。TBSの刺激強度は，筋収縮時運動閾値の80%程度という極めて弱い刺激を用いて実施される。TBSは運動関連領域だけではなく，視覚野[6)]や体性感覚野[7)]，運動前野[8)]などの他の脳領域に対しても効果があることが示唆されている。TBS刺激で誘発されるM1の興奮性変化（可塑性）の生理学的機序に関しては，動物実験で見られる長期増強（Long-Term Potentiation: LTP）・LTDに類似したメカニズムが作用していると考えられている[4)]。

3. 連合性ペア刺激（Paired-Associated Stimulation: PAS）（図2）

「短い時間間隔でシナプス前細胞からの興奮性入力とシナプス後細胞が興奮する対刺激が反復された場合，時間的前後関係に応じて，そのシナプスの伝達効率が増強もしくは減弱する」というHebb理論に基づき，末梢からの電気刺激とTMSを一定の時間差でM1に到達するように組み合わせることで，M1のシナプス結合に可塑的変化が出現することが報告されている[9)]。具体的には，正中神経への電気刺激後25 msに電気刺激と反対側M1へTMS刺激を行うPASを20秒ごと（0.05 Hz）に90ペア（30分）施行することで，M1の興奮性増大が報告されている[9)]。刺激間隔25 msは，電気刺激によるインパルスが皮質感覚野に到達し（20 ms），運動野に与えられる磁気刺激と介在ニューロンを介して皮質で相互作用するタイミングと考えられている。この興奮性増大は体部位局在に従

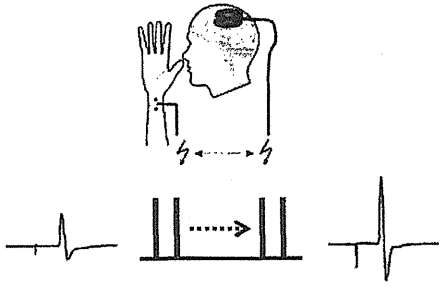

図2　連合性ペア刺激（Paired Associative Stimulation: PAS）
PASの時間（正中神経刺激とTMSによるM1刺激）間隔が25 msのときLTPが誘発され，MEPが増大する。（文献9）より改変して引用）

い，30分以上持続し，抗NMDA剤によって誘発されなくなることから，連合性シナプスLTPと同様の機構が関与していると考えられている[10,11]。一方，刺激間隔を電気刺激の10 ms後に変更することでM1の興奮性が抑制される[11,12]というスパイクタイミング依存的可塑性が報告されている。

PASによるM1可塑性と実際の反復運動によって生じる使用依存的可塑性の間には相互作用がある[13,14]ことも報告されている。これらの結果は，日常生活動作での運動学習や患者での継続的リハビリ治療効果と，PASによって誘導される人為的な可塑性が共通の神経生理学的機序を持つことを示している。

4. 反復4連発磁気刺激法（Quadripulse Stimulation: QPS）

単相性TMSを一定の間隔で4発，5秒毎に30分与える方法である[12]。M1上に与えたQPSの前後でMEPを計測すると，QPS刺激間隔が1.5〜10 msという短時間の場合にはMEP振幅が増大し，30〜100 msと長時間の場合には逆に振幅が小さくなり，その効果は刺激後60分以上持続することが報告されている。これらの結果は，刺激間隔に依存して促通と抑制の二方向性の効果を誘発することができることを示している。また4発の刺激間隔とQPS後の効果との間にはBienenstock–Cooper–Munro（BCM）理論[15]におけるシナプス入力強度とシナプス後電位の変化の関連を示す曲線に類似していることから，動物実験で見られるシナプス可塑性の特性を有していることが示唆されている。

5. 連合性両側刺激（Paried Bilateral Stimulation: PBS）（図3）

両側M1へ7–15 msの時間差でTMSを与えるプロトコルを実施することで，2発目に刺激が与えられる対象側M1の興奮性を増大させるものである。両側半球への刺激回数は0.1 Hzで180回，15分間実施する[16,17]。これは，TMSにより反対側M1に脳梁を介した生じる皮質–皮質間入力と時間的にほぼ一致させるペア刺激を繰り返すことで，標的M1にLTPを誘発する手法である。この低頻度での両側半球を刺激する方法によりM1の興奮性が増大するだけではなく，対側肢の運動機能の向上も報告されている[16]。

6. 運動関連磁気刺激（Movement-Related Cortical Stimulation: MRCS）（図4）

PASやPBSで確認されたLTP様効果を発現させる方法として，運動関連磁気刺激法（MRCS）[12]が報告されている。MRCSは，視覚刺激に対する反応時間課題を行う際に，その運動開始を基準として一定の時間間隔で運動反対側M1にTMSを与える方法である。刺激強度は安静時運動閾値の120%とし，0.2 Hzの頻度で240回（20分間）刺激を繰り返すこの方法は，運動準備や遂行に伴うM1への皮質–皮質間あるいは小脳–視床–皮質の投射入力とTMSを組み合わせるプロトコルと考えられる。運動開始前50 msにTMSを与えるとLTP，100 ms後ではLTDを誘導することが可能であることが報告されている[12]。

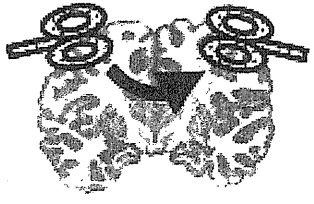

図3　連合性両側刺激（Paired Bilateral Stimulation: PBS）
両側M1を7–15 msの時間差でTMS刺激を繰り返すことで，遅れて刺激された側（2発目）のM1でLTPが誘発される。（文献16）より改変して引用）

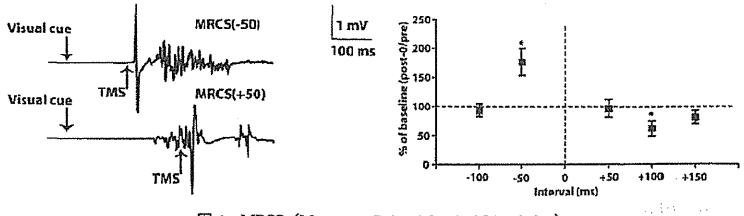

図4　MRCS（Movement-Related Cortical Stimulation）
自発運動とM1へのTMS刺激を組み合わせて刺激する。自発運動開始前50 ms（−50 ms）にTMS刺激を行った際にはLTP，100 ms後（100 ms）ではLTDが誘発される。（文献12）より改変して引用）

7. その他の刺激方法

Khedrら[18]は，閾値下の刺激強度で0.6 Hz，刺激間隔3 msのペア刺激を25分間与えることでM1の抑制効果を示した。一方，Thickbroomら[19]はMEPが1 mVとなる刺激強度で0.2 Hz，刺激間隔1.5 msで30分間の刺激を行うことで，M1の興奮性促通効果を報告している。これらは，短潜時皮質内抑制（Short interval intracortical inhibition）[20]や促通（Short interval intracortical facilitation）[21]に関与する特定の神経回路に対して反復刺激を行う手法である。さらにQuartaroneら[22]は，5 Hzの正中神経刺激と5 HzのM1へのrTMSを刺激間隔25 msで2分間実施するという方法により，M1興奮性を促通し，その効果が6時間持続したことを報告している。これは，PASの変法として対刺激の時間間隔を短くすることで応用しやすくすることを目的とした手法である（Rapid PAS）。

可塑性に関する諸要素

M1を中心とするヒト大脳皮質に可塑性を誘発するTMS手技は上述のように数多く開発され，その効果が報告されている。しかし外部からの非侵襲的脳刺激により可塑性を誘発するためには，刺激条件や被験者の特性など様々な要因が関与することが考えられる。以下にTMSによる脳可塑的変化に関連が深いと考えられる項目について概説する。各種の介入方法の比較検討や臨床応用では，これら諸要素の影響を実験的に統制したり，結果の解釈で考慮したりすることが必要である。

1. 介入中（注意・情動・ドパミン，運動など）

学習や記憶の神経生理学的機序は，神経ネットワーク上のシナプス効率におけるLTPが基盤となっていると考えられている[23,24]。情動に関しては，動物実験において恐怖刺激に対する行動が，扁桃体–視床経路のような感情回路内でLTPの形成を通して獲得されることが報告されている[25,26]。またノルエピネフリンや皮質ステロイド，ドパミンのような神経伝達物質によるLTP変化を通して記憶形成を強化していることも示唆されている[27,28]。一般的に，情動が行動にとって重要であることは広く信じられているが，運動学習と記憶に強く関係する感情と大脳皮質運動関連領域の可塑的変化との関係性はあまり知られていない[29,30]。Koganemaruら[31]は，視覚刺激入力により情動変化を誘発すると共に，TBSを組み合わせる方法によりM1の興奮性変化を検討している。その結果，negativeな情動とTBSの組み合わせによって，M1の興奮性および抑制性回路が共に活性化されたと報告している。さらに標的部位に注意を向けることで，PAS[32]やrTMS[33,34]によるM1可塑的変化が大きくなることから，対象への注意量が可塑性の大きさに影響することが示唆されている。また1 Hzのr TMS前後に持続収縮を行うことで，MEPの抑制効果がなくなることも報告されている[35]。

2. 介入前後（プライミング効果，ホメオスターシス，運動など）

大脳皮質に可塑性を誘発するためには，刺激入力時の状態が大きく関与する。動物実験では，神経活動の記憶がシナプス可塑性を規制する[36,37]ことから，シナプスの強さを選択的に調整できることが報告されている[38]。そして，神経活動は安定性を獲得するためにシナプス強度を動的に調整する恒常性メカニズムによって維持され，その恒常性は皮質ネットワークなど様々な場所で見られることが報告されている[38]。

刺激前に大脳皮質の興奮性を調整する方法としては，プライミング効果を利用する方法があり，大脳皮質へのプライミング効果に関する数多くの先行研究が報告されている[39〜42]。代表的なものとして，Siebnerら[43]は経頭蓋直流電流刺激（tDCS）によるプライミング効果を検討し，1 Hzのr TMSによる効果を両方向性に調整，つまり陽極刺激では1 Hzのr TMS刺激によりMEPの抑制が増大するが，逆に陰極刺激ではMEPを促通することを報告している（図5）。一方高頻度（5 Hz）rTMSによる皮質興奮性を増大させる刺激では，陽極刺激でMEPは抑制され，陰極刺激ではMEPが促通されることが報告されている[41]。さらにM1とその他の運動関連領域との関係性も検討されており，補足運動野[44]や前運動野[45〜47]との連結は運動制御や学習にとって非常に重要であることが報告されている。

また，手指運動や随意筋収縮を刺激前に行うことで，

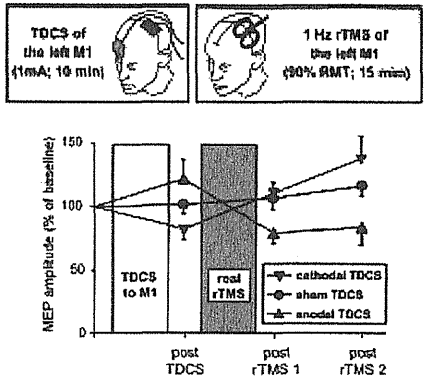

図5 rTMS実施前にtDCSによるプライミング効果の影響を検討

低頻度（1 Hz）rTMSの効果は，rTMS実施前のtDCS陽極刺激により抑制が増強され，逆に陰極刺激でMEP振幅の増大が見られるという，両方向性の調整が出現した。（文献40）より改変して引用）

大脳皮質運動領域の活動性変化を誘発することによりプライミング刺激の代わりとする方法も報告されている。Ziemann ら[14]は，手指外転運動をPAS前に実施することにより，LTP様効果を減弱させる一方，LTD様効果を増大させることを報告している。また，亜最大等尺性収縮をPAS前に実施することでも，LTP様のMEP促通効果を阻害することも報告している[48]。一方，手指トレーニングによる長期効果に関しては，5日目でトレーニングによる逆行性の効果は消失することが報告されている[49]。さらに定期的な有酸素運動が大脳皮質可塑性を調整し[50]，学習や記憶を改善する[51]ことも示唆されている。このように可塑的変化には様々な要因が関係しているが，脳神経由来神経栄養因子（BDNF: Brain-derived neurotrophic factor）の増加[52]に加え，脳血流の増加[53]や血管形成[54]といった要因も含まれていると考えられている。また，これらの報告を支持するようにデスクワーク従事者に比べ活動性の高い人でPASによるLTP変化が大きいことも報告されている[55]。

3. 被験者の特性（遺伝子型，年齢，性別）

性別や遺伝子型など多くの要因による個人差もM1でのシナプス可塑的変化に関与している。近年BDNF遺伝子が，ヒト大脳皮質における非侵襲的刺激による可塑的変化に影響することが明らかとなってきている。Kleim ら[56]は単純な運動課題に関連する神経生理学的変化はval66met多型の保有者で減少することを報告している。また，そうした保有者では，rTMSなどの非侵襲的磁気刺激に対する効果が減少または消失することも報告されている[57]。これら先行研究は，BDNFがヒト運動皮質の可塑的変化に関連していることを示唆している。しかし一方で，QPSにおいてはBDNF対立遺伝子保有者で差は見られないとの報告もあり[58]，BDNF多型の機能的影響はまだ不明な点も残されている。

またヒトは加齢に伴い学習や記憶が低下することはよく知られている。運動皮質に対するPASでは，可塑性の年齢依存現象として高齢者群に比べ若年者または中年者で有意にLTP様反応が高いことが報告されている[59,60]。

性別に関しては，Smith ら[61,62]が海馬での性腺ホルモンレベルの変動を検討し，雌ラットに比べ雄ラットで興奮性レベルが高く，これはテストステロンの興奮性効果であることを報告した。またInghilleri ら[63]はrTMS実験で，エストロゲンレベルと皮質興奮性に正の相関があることを報告している。さらにtDCSの効果に関して，視覚皮質への刺激で女性にのみ陽極刺激で促通効果が出現するという報告や[64]，男性に比べ女性で陰極刺激に対するLTD反応が延長することが報告されている[65]。

臨床応用トピックス

脳卒中後片麻痺患者の運動麻痺に関して，障害側M1からの出力減少，非障害側M1からの過剰な脳梁を経由した抑制により，障害側M1の活動がさらに抑制されているという障害モデルが提唱されている[66]。そして治療として障害側M1の興奮性増大だけではなく，非障害側M1の興奮性低下を促すことが重要とされている。この障害モデルに対して，脳卒中発症後の両側半球間の不均一な活動を調整することを目的として，刺激頻度によって大脳皮質に対する可塑性の作用が異なるrTMSの特性を利用した介入が近年積極的に行われている。

非障害側運動野の興奮性を抑制する方法として臨床で積極的に応用されている低頻度rTMSに関しては，Takeuchi ら[67,68]やFregni ら[69]が，慢性期脳卒中患者に対しrTMS治療群とsham刺激群に分けて比較を行い，非障害側大脳半球M1に1 Hzのを20分程度適用することで，sham群と比べ麻痺側上肢機能の有意な改善が見られたことを報告している。一方，障害側大脳半球の興奮性を高めることを目的とした高頻度rTMSに関しては，Kim ら[3]が脳卒中後3か月経過した患者に対し，高頻度rTMSとsham刺激を実施し，高頻度rTMS群で運動機能の改善が見られたことを報告している。このように低頻度または高頻度刺激において各々効果が報告されているが，Takeuchi ら[68]は低頻度rTMSと高頻度rTMSの効果を直接比較し，高頻度rTMSにおける運動機能改善効果が低いことを報告している。

また近年，Koganemaru ら[70]は慢性期脳卒中患者で特に痙性麻痺を有する患者を対象に，rTMSと運動訓練を併用したハイブリッド型リハビリを提案した。この方法は，上肢屈筋群に過緊張を有する患者に対し，障害側M1への高頻度rTMSと麻痺側上肢の随意的伸展運動訓練を組み合わせる方法で，屈筋群の緊張が抑制され運動機能の向上が得られることを報告している。本法は6週間実施により，終了後2週間以上運動機能改善効果が持続することも報告されており，今後の脳卒中患者に対するリハビリに大きな変化をもたらす可能性がある。

脳卒中以外の疾患に対しても，rTMSの適用が広がっている。うつ病に対するrTMSの効果に関しては，米国において大規模研究が実施され[71~75]，薬剤抵抗性うつ病に対する治療法として承認され，臨床応用が進んでいる。さらにパーキンソン病に対する効果に関しては，高頻度[76~78]または低頻度刺激[79,80]ともに刺激対側の上肢運動機能に向上が見られ，その有効性が報告されている。Hamada ら[81,82]は，パーキンソン病患者の補足運動野に対して5 Hzの高頻度rTMSを8週間実施することで有意な運動機能の改善が見られたことから，補足運動野がパーキンソン病治療の潜在的な刺激領域になり得る可能性を示唆している。また脊髄小脳変性症に対しても，6秒間に1発の低頻度，運動閾値の2.5倍という刺激強度を用いることで歩行能力の改善と小脳・橋の血流増大が報告されている[83]。疼痛に関しては，Katayama ら[84,85]によって難治性慢性疼痛に対する硬膜外電極刺激が治療法の一つとして確立されたことから，磁気刺激による運動野刺激も盛んに行われている。そして，慢性疼痛患者に10～20 Hzの高頻度刺激を実施することで疼痛抑制効果を示したことが報告されている[86,87]。しかし慢性疼痛に対する治療では，硬膜外電極による運動野刺激と同様，rTMS治療の効果がある場合とない場合があると考えられている。

このように，rTMSは非侵襲的に大脳皮質の興奮性を調整できるという大きな利点があり，今後の更なる臨床応用が期待される。そして近年シナプスの可塑的変化に対して計算論的神経科学的手法[88]が適応され，分子レベルからその生理学的機序の解明に取り組まれている。今後の益々の研究の発展により，詳細な神経メカニズムが解明されることが望まれている。

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長ループ反射

美馬達哉

長ループ反射とは

機械的刺激による深部腱反射やperturbationのような筋の素早い伸張あるいは末梢神経の電気刺激によって，当該筋には反射性の活動が誘発される．大きく分けて3つの成分が認められる[1]．脊髄に反射中枢を持ち，単シナプス性反射である短潜時反射(short-latency reflex；SLR，M1成分)，上肢であればSLRの2倍程度の潜時になる長潜時反射(long-latency reflex；LLR，M2成分)，潜時がさらに長くばらつきも大きく，刺激に対して筋収縮させるなどの課題によって増強する随意運動関連成分(M3)である．

筋伸張の場合のもっとも短潜時のM1成分は脊髄に反射中枢を持ち，筋紡錘からのIa求心線維を通じた入力によって誘発される単シナプス性の筋伸張反射である．末梢神経電気刺激の場合は同じ経路の反射弓で誘発されるHoffmann反射に相当する．

LLR(M2)については誘発は容易であるものの，誘発手法や潜時や反射経路によってさまざまな分類法がある．反射中枢は脊髄であるが，多シナプス性だったり，求心経路に違いがあったりするために長潜時となっている(という仮説に立脚する)場合[2]を，短ループ反射と呼ぶ．これに対して，脊髄より中枢側に反射中枢がある(という仮説に立脚する)場合には長ループ反射(long-loop reflex；LLR，長潜時反射と同じ略称である)と呼ばれる．また，LLRは，筋紡錘ではなく皮膚表在覚刺激によっても誘発される[3]．表在覚刺激によって誘発されたLLRは2峰性の筋放電で，促通成分の後に谷のように抑制性の成分があるパターンを示す〔(E1-)I1-E2-I2とも呼ばれる〕．

中枢神経疾患での運動障害や運動異常症では皮質を介した長ループ反射としてのLLRは異常を示すため，さまざまな検討が行われてきた．また，皮質ではない長ループ機構としては脊髄球脊髄反射(spino-bulbo-spinal reflex)がある[4]．

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記録手法

機械的刺激によるLLRの検査は等尺性に筋収縮させた状態でトルクモータなどを用いて，突然の筋伸張を行わせる課題が用いられる(perturbation)[5]．たとえば，長拇指屈筋を弱収縮させた状態で伸張を加えると，潜時20～25ミリ秒でM1成分が認められ，40～50ミリ秒でM2成分(LLR)が認められる．ただし，この手法はトルクモータの性能によって刺激パラメータが変わるため研究施設間での標準化が困難である．

末梢神経電気刺激によってLLRを誘発する手法は通常の筋電計のみでの記録が容易で，標準化しやすい利点がある．上肢の場合は，短拇指外転筋を弱収縮させた状態で運動刺激伝導検査を行うのと同様に，手首部での正中神経刺激(運動閾値上の強度，刺激頻度3 Hz)を行い，整流筋電図を数百回程度加算することで得られる．表在覚刺激によるLLRは橈骨神経の感覚枝を電気刺激(感覚閾値の2～3倍)することで同様に得られる．

Deuschlら[6]によれば，正中神経刺激に対する反応ではH反射(25～34ミリ秒)に続いてLLR-I(40ミリ秒)，LLR-II(50ミリ秒)，LLR-III(75ミリ秒)が認められるという．また，橈骨神経刺激に対してはLLR-I，II，IIIがほぼ同じ潜時で認められるという．ただし，LLR-IIがほぼ全例で認められるのに対して，LLR-IやLLR-IIIの出現率は20%かそれ以下である．

発生機構

ヒトでのLLRは脊髄より中枢側，とくに感覚運動皮質を介した長ループ反射であるという説をもっとも説得力をもって示したのは，Marsdenらによる一連の実験である[7～9]．彼らは筋伸張に対するLLRを多数の多発性硬化症や脳卒中患者で記録し，脊髄後索，感覚運動皮質，皮質脊髄路での局所性障害では，H反射が保たれていてもLLRが消失・減弱ないし遅延することを示し，LLRは経皮質反射(transcortical reflex)であると結論づけた．

LLRを脊髄に反射中枢を持つ短ループであるという説では2つの仮説が知られている．

一つは，筋紡錘の求心線維からのmicroneurogramによる実験結果に基づくもので，末梢の筋への伸張刺激が反響による機械的振動を生じ，Ia線維の反復的な発火を引きおこすことで反復する脊髄反射が生じるという考え方である[10]．

もう一つは，伝導速度の遅い求心線維(group II)の関与する脊髄反射であるという説である．これは，IaとIIの線維をともに刺激する筋伸張がLLRを誘発するのに比べて，Iaを強く刺激する振動刺激を短時間与えてもLLRは誘発されないという観察データに由来している[11]．

以上の諸説のそれぞれに証拠と反証があり，結論は得られていない．臨床的にヒトで観察されるLLRはこれらの混合物である可能性もある．

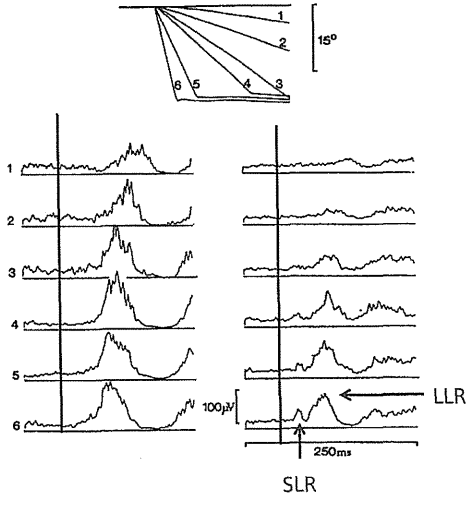

図1　パーキンソン病患者と正常被験者での長潜時反射(LLR)
長拇指屈筋からの記録で，上には角度変化によって与えたperturbationの大きさを示している．健常者では，とくに角速度の大きい4～6の記録で短潜時反射(SLR)と長潜時反射(LLR)を認める．パーキンソン病患者では角速度にかかわらずLLRの増強を認める．(Rothwellら[13]より改変)

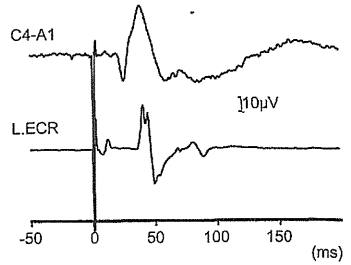

図2　ミオクローヌスでの巨大SEPとC-reflex
良性成人型家族性ミオクローヌスてんかん症例．右正中神経刺激に対するSEPを右中心部C4電極(左耳朶基準電極)にて記録した．SEPは振幅10 μV以上に巨大化しており，同時記録された左橈側手根伸筋(ECR)では安静状態にもかかわらず，潜時41 msでの増強した長潜時反射(LLR)すなわちC-reflexが認められる．

疾患と長ループ反射

LLRが消失・減弱ないし遅延する病態としては，すでに紹介した通り，脊髄後索，感覚運動皮質，皮質脊髄路での障害を伴う疾患(とくに多発性硬化症や脳卒中)である．また，それ以外にはHuntington病で消失することが知られている[12]．これは大脳皮質の広範な機能低下と関連すると考えられる．

LLRが増強する疾患としては，パーキンソン病，皮質基底核変性症，ミオクローヌスを示す疾患などがある．パーキンソン病ではLLRは亢進し，固縮の程度と相関すると考えられている[5]．だが，筋伸張の素早さや筋の近位・遠位による違いもあると報告されている[13](図1)．

LLRの増強が病態そのものと直接に関連しているのがミオクローヌスである[14]．ミオクローヌスてんかんなどに

よる皮質由来のミオクローヌス(皮質性ミオクローヌス)の場合であれば，しばしば安静時であっても，筋仲張ないし末梢神経刺激に対応してLLRが誘発され，これは反射性ミオクローヌスそのものである．

たとえば，手首部での正中神経刺激の場合であれば，刺激後45～60ミリ秒での手内筋での筋放電が観察される(C-reflexと呼ばれる)．この場合，体性感覚誘発電位(SEP)の皮質成分の巨大化も同時に観察される(giant SEP)．これは，感覚運動皮質の興奮性の増大に伴ってSEP増大とLLR増強が生じたものと考えられる(皮質反射性ミオクローヌス)[15](図2)．

皮質基底核変性症では皮質性ミオクローヌスは認めるが巨大SEPはなく，増強したLLRが観察される．また，この増強したLLRの潜時は，ミオクローヌスてんかんでみられるLLRの潜時より短いことが報告されている[16]．また，表在覚刺激でのLLRでは短潜時で増強したE2成分がみられるとともに，抑制性成分が消失する[17]．これらは，電気刺激の求心路が直接に一次運動野に入力するなど，LLRの発生機構そのものが異なっていることによるかもしれない．

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Positive feedback loop via astrocytes causes chronic inflammation in virus-associated myelopathy

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Human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative disease characterized by chronic inflammation in the spinal cord. We hypothesized that a positive feedback loop driven by chemokines may be responsible for the chronic inflammation in HAM/TSP. We aimed to determine the identity of these chemokines, where they are produced, and how they drive chronic inflammation in HAM/TSP. We found that patients with HAM/TSP have extraordinarily high levels of the chemokine CXCL10 (also known as IP-10) and an abundance of cells expressing the CXCL10-binding receptor CXCR3 in the cerebrospinal fluid. Histological analysis revealed that astrocytes are the main producers of CXCL10 in the spinal cords of patients with HAM/TSP. Co-culture of human astrocytoma cells with $CD4^+$ T cells from patients with HAM/TSP revealed that astrocytes produce CXCL10 in response to IFN-γ secreted by $CD4^+$ T cells. Chemotaxis assays results suggest that CXCL10 induces migration of peripheral blood mononuclear cells to the central nervous system and that anti-CXCL10 neutralizing antibody can disrupt this migration. In short, we inferred that human T-lymphotropic virus type 1-infected cells in the central nervous system produce IFN-γ that induces astrocytes to secrete CXCL10, which recruits more infected cells to the area via CXCR3, constituting a T helper type 1-centric positive feedback loop that results in chronic inflammation.

Keywords: HTLV-1; HAM/TSP; CXCL10; CXCR3; astrocyte
Abbreviation: HAM/TSP = human T-lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis

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Introduction

The rise of chronic inflammatory disorders has prompted researchers to reconsider the classical concept of inflammation, which dates back to ancient Roman times when inflammation was first defined as redness, swelling, heat and pain in response to injury or infection. In general, inflammation is an adaptive immune response to tissue malfunction that ideally neutralizes the source of the disturbance and restores tissue homeostasis. Paradoxically, a prolonged state of inflammation has been implicated in the pathogenesis of various diseases characterized by the loss of homeostasis, such as autoimmune diseases, cancers and neurodegenerative diseases (Libby, 2002; Mantovani *et al.*, 2008; Medzhitov, 2008, 2010). To produce effective therapies for these debilitating disorders, we must first elucidate the mechanisms by which this maladaptive chronic inflammatory state develops.

Although there are many chronic inflammatory disorders for which the initiating trigger is ill-defined or unknown, human T-lymphotropic virus type 1 (HTLV-1)-associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a rare neurodegenerative chronic inflammatory disease clearly caused by HTLV-1 retroviral infection (Gessain *et al.*, 1985; Osame *et al.*, 1986). In other words, the HTLV-1-infected cells in patients with HAM/TSP represent a useful starting point from which to investigate the origins of chronic inflammation.

HTLV-1 infects 10–20 million people worldwide, some of whom develop serious conditions such as adult T cell leukaemia (Hinuma *et al.*, 1981) and up to 2–3% of whom develop the debilitating inflammation in the spinal cord that characterizes HAM/TSP (Gessain *et al.*, 1985; Osame *et al.*, 1986). Evidence has accumulated to support the theory that infected $CD4^+$ T cells (as opposed to infected neuronal cells or non-infected peripheral blood mononuclear cells) are primarily responsible for this transition to the HAM/TSP disease state: HTLV-1 primarily infects $CD4^+$ T cells (Richardson *et al.*, 1990); levels of infected $CD4^+$ T cells circulating in the blood of patients with HAM/TSP are higher than those in the blood of asymptomatic carriers (Nagai *et al.*, 1998; Yamano *et al.*, 2002), the levels in the CSF surrounding the spinal cord are higher still (Nagai *et al.*, 2001a); and these infected $CD4^+$ T cells have also been detected in the spinal cord lesions themselves (Moritoyo *et al.*, 1996; Matsuoka *et al.*, 1998). There are many cell types capable of producing an inflammatory response upon contact with viral antigens, and it is true that the cases where these antigen-specific cells are most abundant are indeed in patients with HAM/TSP, but there is a large range of overlap in which patients with HAM/TSP and asymptomatic carriers have the same amount of antigen-specific cells in their peripheral blood mononuclear cells (Jacobson *et al.*, 1990; Jeffery *et al.*, 1999; Kubota *et al.*, 2000; Yamano *et al.*, 2002). Therefore, we hypothesized that their presence may not be the key factor that determines a patient's fate to experience the disease or not, and that perhaps there might be another cell type responsible for initiating the chronic inflammation in HAM/TSP through a more unique pathway. Research shows that infected $CD4^+$ T cells are indeed capable of migrating across the blood–brain barrier into the CNS (Furuya *et al.*, 1997) and secreting proinflammatory cytokines such as interferon-gamma (IFN-γ) (Hanon *et al.*, 2001; Yamano *et al.*, 2005, 2009). We guessed that these cells might even be capable of producing IFN-γ spontaneously due only to intracellular activation of transcription factors by the invading HTLV-1 virus, which has been shown to be capable of such potent effects (Waldmann, 2006).

Studies have indicated that among the $CD4^+$ T cell subtypes, immune responses by $CD4^+$ T helper type 1 (Th1)-like cells may be dominant in patients with HAM/TSP (Goon *et al.*, 2002; Narikawa *et al.*, 2005), leading to the theory that the Th1 axis should be the primary focus in the study of HAM/TSP. These Th1 cells express both the CC chemokine receptor type 5 (CCR5) and CXC motif receptor 3 (CXCR3), which respond to the presence of CC motif ligand (CCL) 3, 4 and 5 and CXC motif ligand (CXCL) 9, 10 and 11, respectively. These ligands are chemokines, a subclass of cytokines that stimulate directed chemotaxis in responsive cells, and it is known that chemokine receptor–ligand interactions play an important role in recruiting immune cells to inflammatory sites (Luster, 1998; Qin *et al.*, 1998). Of particular interest are the CXCR3 agonists, which are regulated by the aforementioned proinflammatory cytokine IFN-γ and carry this relationship in the alternative nomenclature: monokine induced by gamma interferon (MIG/CXCL9), IFN-γ-inducible protein 10 (IP-10/CXCL10), and interferon-inducible T cell alpha chemoattractant (I-TAC/CXCL11) (Proost *et al.*, 2001, 2003). We and others have shown that CCL5, CXCL9, and especially CXCL10, are elevated in the CSF of patients with HAM/TSP (Teixeira *et al.*, 2004; Narikawa *et al.*, 2005; Tanaka *et al.*, 2008; Sato, in press).

We hypothesized that these chemokines play a key role in the pathogenesis of HAM/TSP by recruiting more cells infected with HTLV-1 to the inflammation site and potentially initiating a positive feedback loop. We first compared the levels of several chemokines in the serum and CSF of patients with HAM/TSP and asymptomatic carriers and found that CXCL10 was the most closely associated with known features of HAM/TSP pathogenesis, namely increased CSF cell count. We then analysed samples of peripheral blood mononuclear cells and CSF cells along with images of the spinal cord tissue to demonstrate that $CD4^+$ cells expressing CXCL10-binding CXCR3, namely cells of the Th1 subtype, are indeed infected with HTLV-1, do migrate across the blood–brain barrier into the CNS, and do produce IFN-γ in patients with HAM/TSP. We demonstrated that this IFN-γ production can occur in the absence of external stimuli. Immunohistochemical analysis of the spinal cord tissue not only confirmed that CXCL10 production is elevated in patients with HAM/TSP but also revealed that astrocytes may be the main producers of CXCL10 in the spinal cord. We used novel techniques to demonstrate that these astrocytes likely represent the missing piece of the puzzle in the positive feedback loop: infected $CD4^+$ T cells produce IFN-γ, which stimulates astrocytes to produce CXCL10, which recruits more $CD4^+CXCR3^+$ Th1 cells to the CNS. Finally, chemotaxis assays were used to compare the inhibitory potentials of anti-CXCL10 and anti-CXCR3 neutralizing antibodies on this positive feedback loop as the first step toward the development of an effective therapy.

Materials and methods

Patient selection and sample preparation

Written informed consent was obtained from all patients before the study, which was reviewed and approved by the Institutional Ethics Committee (St. Marianna University) and conducted in compliance with the tenets of the Declaration of Helsinki. The study included 26 HTLV-1 non-infected healthy donors (14 females and 12 males; mean age, 49 years), 29 asymptomatic carriers (21 females and eight males; mean age, 50 years), 17 patients with adult T cell leukaemia with no history of chemotherapy (eight females and nine males; mean age, 68 years), and 58 patients with HAM/TSP (47 females and 12 males; mean age, 62 years). Diagnosis of adult T cell leukaemia was based *on the criteria established by Shimoyama (1991).* HTLV-1 seropositivity was determined by a particle agglutination assay (Serodia-HTLV-1) and confirmed by western blot (SRL Inc.). HAM/TSP was diagnosed according to WHO guidelines (Osame, 1990).

Samples of peripheral blood mononuclear cells were prepared using density gradient centrifugation (Pancoll; PAN-Biotech) and viably cryopreserved in liquid nitrogen with freezing medium (Cell Banker 1; Mitsubishi Chemical Medience Corporation). Plasma and serum samples were obtained from 16 healthy donors, 26 asymptomatic carriers, 30 patients with HAM/TSP and 14 patients with adult T cell leukaemia (six smouldering type and eight chronic type). Multiple serum and CSF samples were taken within a 1-h window for each of 32 patients with HAM/TSP. A Fuchs–Rosenthal chamber (Hausser Scientific Company) was used for CSF cell counts, after which the cells were isolated by centrifugation and cryopreserved in the aforementioned freezing medium. A medulla oblongata tissue sample from one patient with HAM/TSP as well as thoracic spinal cord tissues from four patients with HAM/TSP and six control individuals with no spinal cord lesions (numbered controls 1–6; one female and five males; mean age, 67 years) were obtained post-mortem, fixed in 10% formalin, and embedded in paraffin. Clinical characteristics of the patients with HAM/TSP who underwent post-mortem examination are shown in *Supplementary Table* 1.

Cell culture

Before culture, peripheral blood mononuclear cells from patients with HAM/TSP, asymptomatic carriers and healthy donors were sorted using MACS beads (Miltenyi Biotec) according to the manufacturer's instructions; *CD4$^+$ T cells and CD8$^+$ T cells* were separated negatively, and CD14$^+$ cells were separated positively, and the purity of all cell populations exceeded 95%. The isolated cells were seeded at 1×10^5 cells/200 µl/well in 96-well round-bottom plates in RPMI 1640 medium (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated human serum (Wako Pure Chemical Industries Ltd.), and 1% penicillin/streptomycin antibiotic solution (Wako Pure Chemical Industries Ltd.) *without any stimuli. The culture supernatants* were collected after incubating at 37°C for 24, 48 and 72 h in 5% CO_2.

U251 human astrocytoma cells were cultured in Dulbecco's minimal essential medium (Wako Pure Chemical Industries Ltd.) supplemented with 10% heat-inactivated foetal bovine serum (Gibco-Invitrogen) and 1% penicillin/streptomycin. In total, 2×10^4 U251 cells were then co-cultured in 48-well flat-bottom plates at 37°C for 48 h in 5% CO_2 with 0, 2×10^2, 2×10^3 or 2×10^4 CD4$^+$ T cells isolated from peripheral blood mononuclear cells of patients with HAM/TSP or healthy donors using MACS beads. A control group of 2×10^4 CD4$^+$ T cells was single-cultured under the same conditions. The U251 cells were also cultured with and without 1 ng/ml recombinant human IFN-γ (285-IF, R&D Systems). After culture for 48 h, CD4$^+$ T cells were removed by washing with PBS and the U251 cells were then cultured for an additional 24 h before collecting the culture supernatants.

For the experiment investigating the inhibitory potential of neutra*lizing antibodies, 2×10^4 CD4$^+$ T cells isolated from peripheral blood* mononuclear cells of patients with HAM/TSP using MACS beads were cultured in 96-well round-bottom plates for 72 h under the same conditions, and the culture supernatant was collected after centrifugation. Then, in this supernatant, 2×10^4 U251 cells were cultured in 48-well flat-bottom plates with 10 µg/ml monoclonal neutralization antibodies: anti-IFN-γ antibody (MAB285, R&D Systems), anti-tumour necrosis factor (TNF)-α antibody (MAB610, R&D Systems), or isotype control antibody (MAB002 and MAB003, R&D Systems). The U251 cells were cultured for additional 24 h before collecting the culture supernatants for assay.

Measurement of chemokines, IFN-γ, IL-17A and sIL-2 receptor

The concentrations of four chemokines (CCL4, CCL5, CXCL9 and CXCL10) in the serum and CSF samples and levels of CXCL10, IFN-γ and IL-17A in the culture supernatants were measured with a cytometric bead array kit (BD Biosciences) using a FACSCalibur flow cytometer (BD Biosciences) according to the manufacturer's instructions. It should be noted that the cytometric bead array kit measures the total concentrations of all chemokine isoforms irrespective of aminoterminal variation (Proost *et al.*, 2001, 2003). The sIL-2R in the serum was measured using an ELISA (Cell-free N IL-2R, Kyowa Medex).

Flow cytometric analysis

Peripheral blood mononuclear cells and CSF cells, which were obtained on the same day, were immunostained with various combinations of the following fluorescence-conjugated antibodies: anti-CD3 (UCHT1), anti-CD4 (OKT4), anti-CD8 (RPA-T8), anti-CD19 (HIB19), anti-CD14 (61D3) (all from eBioscience), and anti-CXCR3 (1C6; BD Biosciences). The cells were stained with a saturating concentration of antibody in the dark (4°C, 30 min) and washed twice before analysis using FACSCalibur (BD Biosciences). Data were processed using FlowJo software *(TreeStar). For cell sorting, JSAN (Bay Bioscience,) was used,* and the purity exceeded 95%.

Real-time polymerase chain reaction

The HTLV-1 proviral DNA load was measured using ABI Prism 7500 SDS (Applied Biosystems) as described previously (Yamano *et al.*, 2002). In brief, DNA was extracted and 100 ng samples were analysed per well. The proviral DNA load was calculated using the following formula: copy number of HTLV-1 (*pX*) per 100 cells = (copy number of *pX*) / (copy number of β-actin / 2) × 100.

Tissue staining

Formalin-fixed thoracic spinal cord and medulla oblongata tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols and distilled water. The antigenicity of the tissue sections was recovered using a standard microwave heating technique. For immunohistochemistry, the slides were incubated with anti-CXCL10/IP-10 antibody, followed by detection with streptavidin–biotin–horseradish peroxidase and diaminobenzidine (DakoCytomation Japan Co. Ltd.). The CXCL10$^+$ cells in the spinal cord were also counted under the microscope; the data show the mean number of cells in three random 1-mm^2 fields per sample. Haematoxylin and eosin staining was conducted to detect inflammatory cells that had invaded the tissue samples. For immunofluorescence (thoracic spinal cord sections only), the slides were incubated in phosphate-buffered saline with 10% goat serum for 1 h at room temperature, in anti-CXCR3 antibody (Abcam), anti-CXCL10/IP-10 antibody (Santa Cruz Biotechnology), and anti-glial fibrillary acidic protein (GFAP) antibody (DakoCytomation Japan Co. Ltd) overnight at 4°C, labelled with Alexa Fluor® 488 or Alexa Fluor® 594 conjugated secondary antibody (Invitrogen), and *examined under a fluorescence microscope (Nikon* eclipse E600 with fluorescence filter Nikon F-FL; Nikon Instech) with rabbit or mouse immunoglobulin G (IgG) as the negative control.

Chemotaxis assay

Peripheral blood mononuclear cells from patients with HAM/TSP were washed and then suspended (at 1×10^7 cells/ml) in 37°C serum-free RPMI 1640 medium containing 1 mg/ml bovine serum albumin (Wako Pure Chemical Industries, Ltd.), hereafter 'chemotaxis medium'. The lower wells of a 96-well chemotaxis chamber (MBA96; Neuroprobe) were filled with chemotaxis medium containing 0.25 µg/ml recombinant human CXCL10 protein (266-IP; R&D Systems). For the negative control, the lower wells were filled with only the chemotaxis medium. For chemotaxis assays using neutralizing monoclonal antibodies, peripheral blood mononuclear cells were pretreated (room temperature, 30 min) with 10 µg/ml of anti-CXCL10 antibody (MAB266; R&D Systems), 10 µg/ml of anti-CXCR3 antibody (MAB160; R&D Systems), or 10 µg/ml of isotype control antibody (MAB002; R&D Systems). A polyvinylpyrrolidone-free micropore polycarbonate filter (PFD5; Neuroprobe) with 5-µm pores was placed over the lower chamber. The upper wells were filled with 1×10^6 peripheral blood mononuclear *cells in 100 µl of chemotaxis medium. The chamber was incubated* for 120 min at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the fluid in the lower chambers was collected and cell counts were determined using FACSCalibur. To compare results across all chemotaxis assays, a chemotactic index was calculated using the following formula (Nie *et al.*, 2009):

$$\text{Chemotactic index} = (\text{number of migrated cells in a test sample well}) / (\text{number of migrated cells in a negative control well})$$

To determine the inhibitory effect of neutralizing antibodies, an inhibitory efficiency scale was calculated using the following formula:

$$\text{Inhibitory efficiency (\% inhibition)} = \{[(\text{chemotactic index of isotype control}) - 1] - [(\text{chemotactic index of neutralizing antibody}) - 1]\} / [(\text{chemotactic index of isotype control}) - 1] \times 100$$

Proliferation assay

The migrated cells in the lower chamber after the chemotaxis assay were collected and washed with RPMI 1640 medium supplemented with 5% foetal bovine serum and 1% penicillin/streptomycin. Those cells were then plated on 96-well round-bottom plates and cultured in the same medium without any mitogenic stimuli in 5% CO_2 at 37°C. Cell proliferation was measured using a ^{3}H-thymidine incorporation assay as described previously (Yamano *et al.*, 2009).

Statistical analysis

Correlation analysis was assessed using Spearman's rank test. The paired *t*-test was used for within-group comparisons, and the *t*-test or the Mann–Whitney *U*-test was used for comparisons between groups. One-way ANOVA was used for multiple comparisons followed by Tukey's test. The Friedman test was used for paired multiple comparisons, followed by the Dunn test. Statistical analyses and graphs were performed using Graphpad Prism 5 and Prism statistics (GraphPad Software, Inc), and statistical significance was set at $P < 0.05$.

Results

Significantly higher levels of cerebrospinal fluid CXCL10 compared with serum CXCL10 in patients with HAM/TSP

To determine whether the aforementioned chemokines were involved in the migration of cells to the CNS, we first compared the levels of these chemokines with CSF cell counts in patients with HAM/TSP ($n = 29$). CSF cell counts significantly correlated with levels of CXCL10 and CXCL9 but not with those of CCL5 or CCL4, the negative control (Fig. 1A). In addition, the correlation was clearly stronger with CXCL10 than with CXCL9. Following this, we compared the CSF and serum levels of these chemokines. Interestingly, only CXCL10 levels were higher in the CSF than the serum, although serum CXCL10 levels were also high to some extent (Fig. 1B, $P < 0.0001$). Next, to investigate whether these high CXCL10 levels were a HAM/TSP-specific phenomenon within HTLV-1-associated disorders, we tested for a correlation between CXCL10 and soluble interleukin-2 receptor (sIL-2R), a marker for adult T cell leukaemia (Yasuda *et al.*, 1988). As expected, serum sIL-2R levels were the highest in patients with adult T cell leukaemia. By contrast, plasma CXCL10 levels were significantly higher in patients with HAM/TSP than in those with adult T cell leukaemia, asymptomatic carriers or healthy donors (Supplementary Fig. 1A). This higher concentration of plasma CXCL10 in patients with HAM/TSP was observed even when compared to asymptomatic carriers with equivalently high proviral loads (Supplementary Fig. 1B).

Existence of abundant CXCR3$^+$ cells in the spinal cords of patients with HAM/TSP

Because CXCL10 is a ligand of CXCR3, we investigated the possibility of CXCL10 recruiting proinflammatory CXCR3$^+$ cells into the CSF by measuring the presence of CXCR3$^+$ cells in the CSF and spinal cord lesions of patients with HAM/TSP (Fig. 2A–C). Flow cytometric analysis revealed that the average percentage of

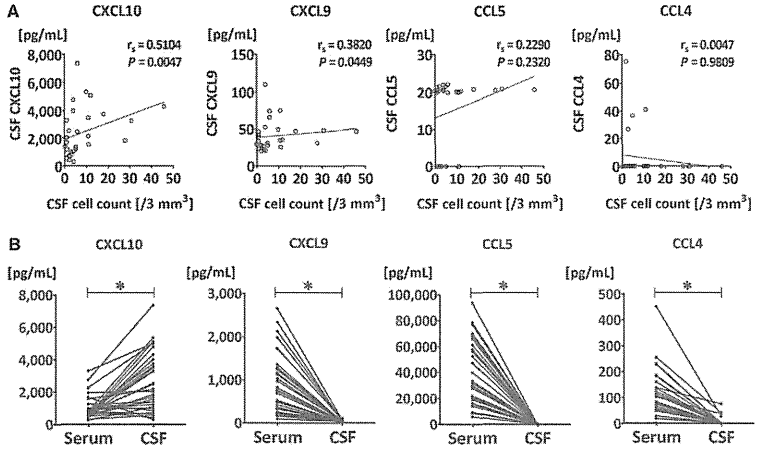

Figure 1 CXCL10 levels in CSF of patients with HAM/TSP were correlated with CSF cell counts and were significantly higher than those in serum of patients with HAM/TSP. (**A**) Correlation analysis between CSF levels of four chemokines (CXCL10, CXCL9, CCL5 and CCL4) and CSF cell counts in patients with HAM/TSP ($n = 29$). Statistical analysis was performed using Spearman's rank test. The linear regression line is indicated by a straight line in each graph. (**B**) Comparison of concentrations of four chemokines (CXCL10, CXCL9, CCL5, and CCL4) in CSF and serum samples obtained from patients with HAM/TSP such that all samples from a given patient were taken within a 1-h window of the first sample taken from that patient ($n = 32$). *$P < 0.0001$ by the paired t-test.

CXCR3$^+$ cells among CSF cells was 92.4 ± 7.0%, whereas the average percentage of CXCR3$^+$ cells among peripheral blood mononuclear cells was 9.9 ± 8.2% ($P < 0.0001$, Fig. 2B). Immunofluorescence staining revealed abundant CXCR3$^+$ cell infiltrate around small vessels in the leptomeninges of spinal cord lesions in patients with HAM/TSP (Fig. 2C). We examined the types of CXCR3$^+$ cells in the CSF using flow cytometry and found that CSF CXCR3$^+$ cells mainly consist of CD3$^+$ cells (>90%) and small populations of CD14$^+$ and CD19$^+$ cells (Fig. 2D, left). Uniquely, the percentage of CXCR3$^+$ cells was extremely high in all CSF cell populations under study, especially CD4$^+$ (94.33 ± 2.95%), CD8$^+$ (98.64 ± 1.05%), and even CD14$^+$ (84.97 ± 18.49%) and CD19$^+$ (76.38 ± 17.35%) cells (Supplementary Fig. 2). Our data show that the ratio of CD4$^+$ to CD8$^+$ cells in the CSF was ~1:1 in patients with HAM/TSP (Fig. 2D, right). In both these cell populations, the rate of CXCR3 positivity was higher in CSF cells than in peripheral blood mononuclear cells (Supplementary Fig. 2). The percentage of CXCR3$^+$ cells in peripheral blood mononuclear cells of patients with HAM/TSP was lower than those in peripheral blood mononuclear cells of asymptomatic carriers as well as healthy donors; however, there were no significant differences between patients with adult T cell leukaemia and patients with HAM/TSP (Supplementary Fig. 3A). This lower percentage of CXCR3$^+$ cells in patients with HAM/TSP was observed even when compared with asymptomatic carriers with equivalently high proviral loads (Supplementary Fig. 3B). Finally, to support our hypothesis that HTLV-1-infected T cells (the majority of which are known to be CD4$^+$) migrate from the circulating blood to the spinal cord tissue through CXCL10–CXCR3 interaction, we confirmed that there does exist a subset of peripheral CD4$^+$CXCR3$^+$ T cells infected with HTLV-1 (Fig. 2E).

Numerous CXCL10-producing cells in inflamed spinal cords of patients with HAM/TSP

To quantitatively compare the level of expression of CXCL10, we microscopically counted the number of CXCL10$^+$ cells in the spinal cord tissue and found a larger number of CXCL10$^+$ cells in the spinal cord lesions of patients with HAM/TSP than in control patients (Fig. 3A, $P = 0.0095$). In addition, we compared tissue sections from the thoracic spinal cord (a region of high inflammation) and the medulla oblongata (comparatively very low inflammation) from a single patient with HAM/TSP, and we observed a much larger CXCL10 presence in the thoracic spinal cord region (Supplementary Fig. 4).

Astrocytes as the main producers of CXCL10 in the spinal cords of patients with HAM/TSP

To identify which cell populations are the main CXCL10 producers, we immunostained thoracic spinal cord tissues from patients with

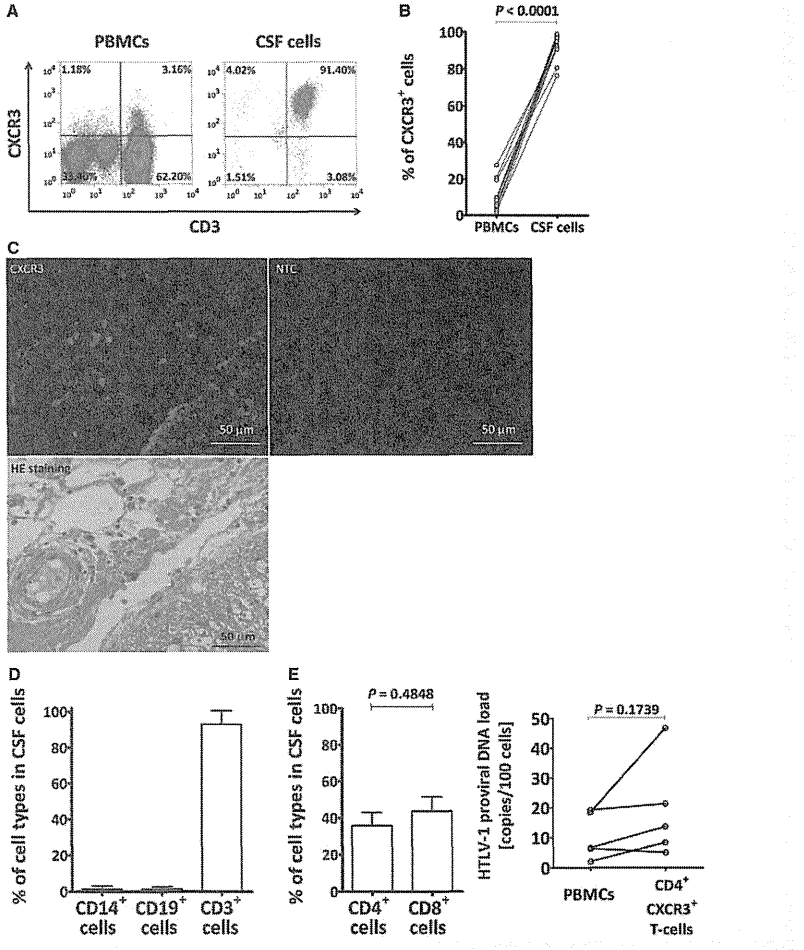

Figure 2 Abundant CXCR3$^+$ cells in the CSF and spinal cord tissue of patients with HAM/TSP. (**A**) Representative dot plots of CD3 and CXCR3 expression in peripheral blood mononuclear cells (PBMCs, *left*) and CSF cells (*right*) from a patient with HAM/TSP measured using flow cytometry. (**B**) Comparison of the percentages of CXCR3$^+$ cells in peripheral blood mononuclear cells and CSF cells, samples of which were obtained from 12 patients with HAM/TSP such that all samples from a given patient were taken within a 1-h window of the first sample taken from that patient. Statistical analysis was performed using the paired t-test. See also Supplementary Fig. 2. (**C**) Representative images of immunofluorescent detection of CXCR3, shown in green (*upper* panels), and haematoxylin-eosin (HE) staining for inflammatory cells, shown in blue (*lower* panel), in the thoracic spinal cords of patients with HAM/TSP. Rabbit IgG antibody used as the negative control (NTC). (**D**) *Left*: Percentages of CD3$^+$, CD19$^+$, and CD14$^+$ cells in CSF cells derived from patients with HAM/TSP ($n = 6$). *Right*: Percentages of CD4$^+$ cells and CD8$^+$ cells. Statistical analysis was performed using the Mann–Whitney U-test. Error bars represent the mean ± SD. (**E**) The HTLV-1 proviral DNA loads of CD4$^+$CXCR3$^+$ T cells with peripheral blood mononuclear cells as the control. This result confirms the non-negligible existence of HTLV-1-infected CD4$^+$CXCR3$^+$ T cells, which may migrate to the CNS. Cells are from patients with HAM/TSP ($n = 5$). Statistical analysis was performed using the paired t-test.