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Neuroscience Letters
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Short-interval intracortical inhibition is modulated by high-frequency peripheral mixed nerve stimulation

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Received 26 January 2007; received in revised form 9 April 2007; accepted 12 April 2007

Abstract

Cortical excitability can be modulated by manipulation of afferent input. We investigated the influence of peripheral mixed nerve stimulation on the excitability of the motor cortex. Motor evoked potentials (MEPs), short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) in the right abductor pollicis brevis (APB), extensor carpi radialis (ECR) and first dorsal interosseous (FDI) muscles were evaluated using paired-pulse transcranial magnetic stimulation (TMS) before and after high-frequency peripheral mixed nerve stimulation (150 Hz, 30 min) over the right median nerve at the wrist. The MEP amplitude and SICI of the APB muscle decreased transiently 0–10 min after the intervention, whereas the ICF did not change. High-frequency peripheral mixed nerve stimulation reduced the excitability of the motor cortex. The decrement in the SICI, which reflects the function of $GABA_A$ergic inhibitory interneurons, might compensate for the reduced motor cortical excitability after high-frequency peripheral mixed nerve stimulation.

Keywords: Peripheral mixed nerve stimulation; Transcranial magnetic stimulation; Motor excitability; Plasticity; Short-interval intracortical inhibition; $GABA_A$ergic inhibitory interneurons

Afferent peripheral somatosensory input has been shown to alter the excitability of the sensorimotor cortex in animal [15] and human studies [18]. For example, transcutaneous electrical nerve stimulation (TENS), the technique of continuous peripheral electrical stimulation at a sub-motor threshold intensity, has been used as a method for chronic pain relief [2,7,22]. It was reported that TENS caused changes in the sensory system, including a decrement in somatosensory-evoked potentials [22] and pain-related evoked potentials [7]. TENS also affects the motor cortex; high-frequency TENS transiently reduced motor evoked potentials (MEPs) [14,21]. Other studies found that prolonged peripheral electrical nerve stimulation led to the increment of MEPs [9,17,18]. There is a hypothesis that these discrepancies of the effects of peripheral somatosensory input on the motor cortical excitability might depend on the frequency of sensory stimulation [3]. In the present study, to clarify the mechanism underlying the alteration in excitability in the motor cortex after somatosensory input, we studied the effects of high-frequency peripheral mixed nerve stimulation on the motor cortical excitability using a paired-pulse stimulation technique [12].

We studied 11, right-handed, healthy volunteers (7 women), 20–39 years of age (mean, 27.1 ± 4.8 years of age). None had a history of physical or neurological illness. This study was approved by the Human Ethics Committee of Tottori University and was carried out in accordance with the Declaration of Helsinki. All participants gave their informed consent prior to participation.

Subjects lay supine on a bed during the experiment. Electrical stimuli were delivered to the right median nerve at the wrist through a pair of 2 × 2-cm Ag–AgCl disposable surface electrodes (cathode proximal). The current frequency was set at 150 Hz, the pulse width was set at 100 μs, and the stimulus was delivered in a symmetrical rectangular biphasic waveform. The stimulus strength was 6.8–9.5 mA (mean 8.0 ± 1.2 mA) and produced a tingling sensation in the stimulated area without muscle twitch or pain. Stimuli were administered for 30 min in 2-s trains at 150 Hz (300 stimuli/train), separated by 2-s pauses. The effects of peripheral mixed nerve stimulation on the motor cortical excitability were examined.

Transcranial magnetic stimulation (TMS) was performed using a round coil (external diameter, 130 mm) connected to a

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0304-3940/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2007.04.059

Magstim 200 stimulator (Magstim Co., Whitland, Dyfed, UK). The coil was positioned over the vertex in the optimal scalp position that would elicit motor responses in the right abductor pollicis brevis (APB) muscle. The resting motor threshold (RMT) was determined according to the recommendation of an International Federation of Clinical Neurophysiology (IFCN) Committee [20] and was defined as the intensity of stimulation that elicits at least five MEPs of 50 μV in 10 trials from the right APB muscle. The MEP amplitude was measured using the stimulator intensity sufficient to evoke a peak-to-peak amplitude of 2 mV of the MEP in the relaxed APB. Short-interval intracortical inhibition (SICI) and intracortical facilitation (ICF) were measured using the paired-pulse method, as described previously [12]. The SICI was evaluated at an interstimulus interval (ISI) of 3 ms, and the ICF was evaluated at an ISI of 15 ms. The conditioning stimulus intensity was set at 80% RMT for both SICI and ICF. The test stimulus intensity was set at a peak-to-peak amplitude of 2 mV of the MEP in the relaxed APB. The stimulation rate was about 0.1 Hz, and it took about 10 min to record 60 trials for three parameters (20 trials for each parameter were recorded and averaged). The order of presentation of MEP (test stimulation only), SICI (ISI = 3 ms) and ICF (ISI = 15 ms) intervals was controlled to be presented randomly by a computer program (SPIKE2, CED, Cambridge, UK). MEP, SICI and ICF were recorded from the APB, the extensor carpi radialis (ECR) and the first dorsal interosseous (FDI) muscles by a pair of 2 × 2 cm Ag–AgCl disposable surface electrodes in a belly-tendon montage. The electromyogram was recorded from a pair of electrodes and filtered (50–200 Hz), then digitized with an analogue-to-digital converter (micro1401, CED, Cambridge, UK) at a sampling rate of 10 kHz and stored on a personal computer. Each parameter was measured before, 0–10, 15–25, 30–40, 45–55 and 60–70 min after the peripheral mixed nerve stimulation session.

Statistical analysis was carried out using two-way repeated measure analyses of variance (ANOVA) with time (before, 0–10, 15–25, 30–40, 45–55 and 60–70 min after mixed nerve stimulation – 6 levels) and muscles (APB, ECR and FDI – 3 levels) as factors. The persistence of the effect of mixed nerve stimulation was studied in each of the three muscles using a one-way repeated measure ANOVA, with time (6 levels) as a factor. If the effect was significant, a post hoc Dunnett's paired t test was performed on the data. A value of $P < 0.05$ was considered to be statistically significant.

All subjects felt tingling but not pain in the stimulated and affected area during intervention and a few minutes after the end of intervention.

When the three muscles were compared, the factor of time was different for the MEP amplitude and SICI (MEP: $F_{(5, 192)} = 3.143$; $P = 0.024$, SICI: $F_{(5, 192)} = 2.700$; $P = 0.023$), whereas there was no difference for the ICF ($F_{(5, 192)} = 1.861$; $P = 0.136$). Two-way repeated measure ANOVA with time × muscles showed no significant differences in the MEP amplitude, SICI and ICF (MEP: $F_{(5, 192)} = 0.653$; $P = 0.762$, SICI: $F_{(5, 192)} = 0.979$; $P = 0.473$, ICF: $F_{(5, 192)} = 0.682$; $P = 0.736$). Changes in the MEP, SICI and ICF after intervention did not differ statistically among the three muscles. Concerning the MEP amplitude, a significant change was observed in the APB with time by one-way repeated measure ANOVA ($F_{(5, 60)} = 5.531$; $P = 0.041$), whereas neither the ECR nor the FDI showed statistically significant changes (Fig. 1(A and B)). Post hoc analysis revealed that the MEP of the APB was reduced significantly 0–10 min after intervention (1.18 ± 0.23 mV) compared to the pre-intervention (1.92 ± 0.46 mV) ($P = 0.040$). One-way repeated measure ANOVA revealed a significant effect in the SICI of the APB with time ($F_{(5, 60)} = 4.667$; $P = 0.044$), whereas neither the ECR nor the FDI showed statistically significant changes (Fig. 2(A and B)). Post hoc analysis revealed that the SICI of the APB was reduced significantly 0–10 min after intervention compared to the pre-intervention ($P = 0.047$). Although single-pulse MEPs decreased after intervention, paired-pulse MEPs increased after intervention (pre-intervention: 0.56 ± 0.11 mV; 0–10 min: 0.81 ± 0.23 mV), as a result SICI was reduced significantly (Fig. 2(A)).

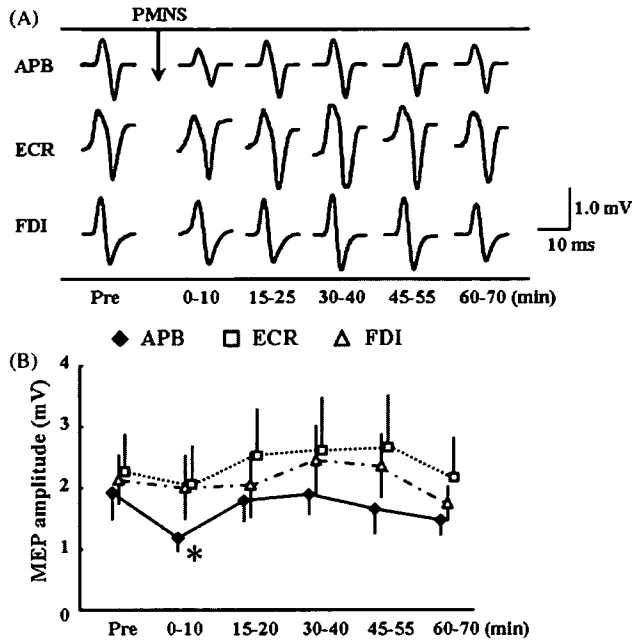

Fig. 1. (A) Effects of peripheral mixed nerve stimulation on the MEP amplitudes of the right APB, ECR and FDI muscles of averaged waveforms. The MEP amplitude of the APB muscle decreased 0–10 min after peripheral mixed nerve stimulation. (B) Time course of the changes in the MEP amplitudes of the right APB, ECR and FDI muscles. The MEP amplitude of the APB muscle was reduced significantly after peripheral mixed nerve stimulation compared to that pre-intervention (asterisk). PMNS: peripheral mixed nerve stimulation.

In the present study, we revealed that not only the MEP amplitude but also the SICI of the APB muscle decreased after high-frequency peripheral mixed nerve stimulation was delivered to the median nerve territory, and these effects did not continue for more than 15 min. There was no effect of mixed nerve stimulation on the other muscles analyzed.

There is a consensus that afferent somatosensory input influences not only the sensory cortex but also the motor cortex. Vibratory input from muscle modulated the motor cortical excitability [19]. Electrical pharyngeal stimulation improved swallowing behavior in dysphagic stroke patients and facili-

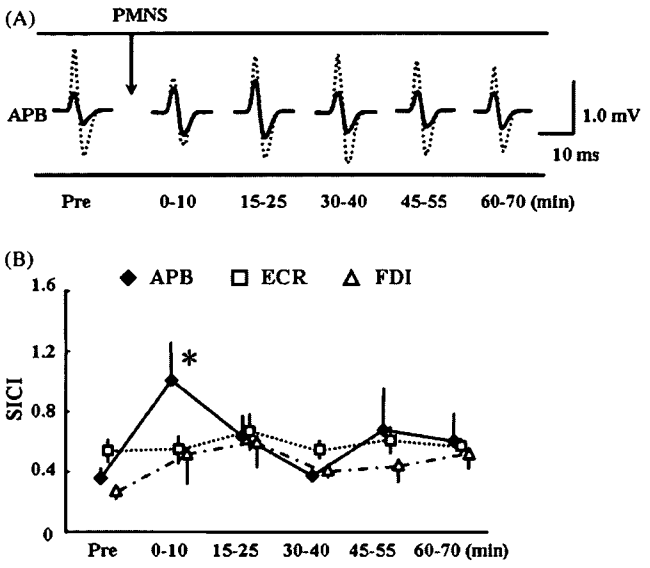

Fig. 2. (A) SICI of the right APB muscle in averaged waveform, before and after peripheral mixed nerve stimulation. Paired-pulse MEPs (continuous line) after the intervention (0.81 ± 0.23 mV) were larger than those pre-intervention (0.56 ± 0.11 mV). Single-pulse MEPs (dotted line) decreased after the intervention. (B) Time course of the changes in the SICI of the right APB, ECR and FDI muscles. The SICI of the APB muscle was reduced significantly after peripheral mixed nerve stimulation (asterisk); thereafter, it recovered to the baseline level. PMNS: peripheral mixed nerve stimulation.

tated pharyngeal MEP, and magnetoencephalography revealed activation in the sensorimotor cortex by pharyngeal stimulation [4]. Since TMS studies confirmed the MEP modulation after peripheral mixed nerve stimulation, it is thought that peripheral mixed nerve stimulation can affect the motor cortical excitability. In addition, the MEP changes depended on the stimulus frequency. Some reports suggested that high-frequency (>10 Hz) stimulation decreased the motor excitability [14,21], whereas low-frequency (<10 Hz) stimulation increased it [5,9,17,18]. Since a high-frequency peripheral mixed nerve stimulation paradigm was employed in the present study, we assumed that the transient decrement in the MEP that we observed was caused by the reduction in the motor cortical excitability, and this result was in line with those of previous studies [14,21]. Furthermore, previous studies demonstrated that neither peripheral mixed nerve stimulation nor TENS produced a significant change at the muscle, the neuromuscular junction and the peripheral and spinal neurons, because there was no change in M-responses, H-waves and F-waves after intervention [17,21]. Therefore, the MEP changes after peripheral mixed nerve stimulation could have occurred as a result of the changes in the motor cortical excitability. The mechanisms of the changes in the motor cortical excitability might be related to the functional changes in plasticity in the motor cortex, because the MEP reduction appeared transiently after peripheral mixed nerve stimulation and was reversible. We could speculate that the afferent input might have reached the motor cortex via the thalamo-cortical pathway [8] or via a cortico-cortical connection from the somatosensory cortex [10,11,16].

The inhibitory effect on the APB muscle in the present study was shorter than that on the forearm flexor muscle in the previous study [21]. One reason was that the stimulus intensity of the intervention in the present study was weaker than that in Tinazzi's study (in the present study 6.8–9.5 mA, in Tinazzi's study 11–16 mA). Subjects felt pain in the median nerve-innervated area when stronger stimulation as in Tinazzi's study was delivered, because we performed not muscle stimulation but median nerve stimulation. We supposed that different stimulus intensities might reflect the dissimilar durations of the effects between the two studies. Another reason was that the stimulus sites were different between the two studies (TENS was delivered to the forearm flexor muscles in Tinazzi's study, electrical nerve stimulation was delivered to the median nerve at the wrist in the present study). A previous study reported that a long period of ulnar nerve electrical stimulation at the wrist (10 Hz, 2 h) increased MEPs in the abductor digiti minimi muscle, and this effect disappeared within 20 min [9]. The effects of peripheral nerve stimulation spread in the innervated territory, and innervated muscles were affected secondarily, whereas TENS stimulates muscles and nerves directly. These methodological differences might explain the dissimilar durations of the effects for the motor cortical excitability corresponding to the target muscle.

The phenomenon of SICI is thought to reflect the activity of $GABA_A$ergic inhibitory interneurons within the motor cortex [1,6,12,23]. The present results showed that SICI in the affected muscle was disturbed transiently after high-frequency peripheral mixed nerve stimulation. This result revealed that $GABA_A$ergic inhibitory interneurons were deactivated in spite of the reduction in excitability in the motor cortex. It was reported that muscle fatigue transiently decreased the MEP and SICI, and the authors speculated that the decrement in the SICI might compensate for the reduced motor cortical excitability after muscle fatigue [13]. Although the detailed mechanisms of the reduction in the SICI might differ between high-frequency peripheral nerve stimulation and muscle fatigue, similar MEP and SICI changes were obtained. That was why we speculated that $GABA_A$ergic inhibitory interneurons might be deactivated to compensate for the reduced motor cortical excitability after high-frequency peripheral nerve stimulation. This hypothesis was compatible with the reasons why peripheral mixed nerve stimulation did not cause muscle weakness. A previous study revealed that low-frequency peripheral mixed nerve stimulation at the wrist produced the increment in the MEPs but no change in the SICI [9]. In this case, the $GABA_A$ergic inhibitory interneurons might not have to activate because the motor cortical excitability did not decrease. Therefore muscle weakness was also not caused in low-frequency intervention.

In summary, we found that high-frequency peripheral mixed nerve stimulation transiently regulated the excitability of the motor cortex with a reduction of SICI. This phenomenon might indicate that high-frequency peripheral mixed nerve stimulation brought the decrement of inhibitory function to compensate for the reduced excitability in the motor cortex. These findings proved that peripheral mixed nerve stimulation can modulate the motor cortex neurophysiologically.

Acknowledgement

This study was supported by a Grant-in-Aid from the Research Committee on rTMS treatment of movement disorders, the Ministry of Health and Welfare of Japan (17231401).

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Clinical Neurophysiology 118 (2007) 1460–1463

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Short latency afferent inhibition is not impaired in mild cognitive impairment

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Accepted 17 March 2007
Available online 25 April 2007

Abstract

Objective: To determine whether cortical cholinergic circuit impairment exists in the mild cognitive impairment (MCI) brain.
Methods: Fifteen healthy elderly controls (NC), 16 amnesic MCI subjects and 12 probable Alzheimer's disease (AD) subjects were recruited. Conditioning stimuli were delivered at the right wrist followed by test transcranial magnetic stimulation (TMS) of the left motor cortex. The center of the linear contiguous segment of the coil was placed over a point 5 cm lateral to the vertex on the interaural line. The interstimulus intervals (ISIs) between the conditioning stimuli and the test stimuli were set at 20, 40, 100, 200 and 600 ms. An inhibitory effect that occurred at ISIs as short as 20 ms was defined as short-latency afferent inhibition (SAI).
Results: SAI was significantly reduced in subjects with AD compared with NC, but it was not reduced in subjects with MCI.
Conclusions: A difference in cortical excitability between subjects with AD and subjects with MCI could be captured by an in vivo neurophysiological method.
Significance: The state of the neurotransmitter systems, including the cortical cholinergic system, is thought by some compensatory mechanisms to be kept at the normal level in subjects with MCI.

Keywords: Afferent inhibition; Dementia; Cortical cholinergic circuit; Transcranial magnetic stimulation; Mild cognitive impairment

1. Introduction

Mild cognitive impairment (MCI) is defined as a level of cognitive functioning that reflects an intermediate state between normal aging and dementia. In the MCI state, subjects experience subtle cognitive deficits with largely intact cognition and activities of daily living (Petersen et al., 2001). The annual conversion rate from MCI to Alzheimer's disease (AD) is approximately 12%, and the 3-year conversion rate is approximately 35% (Petersen et al., 1999). Cholinesterase inhibitors (ChEIs) have been used widely for the treatment of AD and have positive effects on global cognitive function, activities of daily living and behavioral symptoms in AD. These agents were introduced based on the cholinergic hypothesis, which suggests that AD results from a selective loss of cholinergic neurons, decreasing acetylcholine levels in the central nervous system. An intervention to prevent the conversion of MCI to AD would be desirable. Clinical questions are whether MCI involves a loss of cholinergic neurons and whether it is worthwhile to use ChEIs to improve symptoms in MCI.

Short-latency afferent inhibition (SAI) is a motor-evoked potential (MEP) inhibition produced by a conditioning afferent pulse applied to the median nerve at the wrist approximately 20 ms prior to transcranial magnetic stimulation (TMS) of the hand area of the contralateral motor cortex (Tokimura et al., 2000). SAI is significantly reduced by the anticholinergic agent (muscarinic [M1] antagonist) scopolamine (Di Lazzaro et al., 2000), and is also reduced in AD (Di Lazzaro et al., 2002), whereas it is normal in frontotemporal dementia (Di Lazzaro et al.,

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1388-2457/$32.00
doi:10.1016/j.clinph.2007.03.018

2006). On the basis of these findings, SAI may reflect the function of cortical cholinergic circuits.

To determine whether the reduction of SAI is an early or late change during the progression of cognitive decline, we measured SAI in subjects with MCI and compared it with that measured in subjects with AD.

2. Methods

2.1. Subjects

Fifteen healthy elderly controls (NC), 16 amnesic MCI subjects and 12 probable AD subjects were recruited. Demographic information is presented in Table 1. The diagnosis of amnesic MCI was based on Mayo Clinic criteria: (1) subjective memory complaint; (2) normal activities of daily living; (3) normal general cognitive functioning; (4) abnormal verbal and/or nonverbal memory for age; (5) absence of dementia (Petersen et al., 1999). Since our study was performed from 2003 to 2004, we used these criteria rather than the latest set. The diagnosis of probable AD was based on the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria (Litvan et al., 1996; McKeith et al., 1996; McKhann et al., 1984). All patients underwent MRI scan, routine blood testing, cardiac physiological testing and neuropsychiatric tests to exclude depression, cardiovascular disease and cerebrovascular disease. None of the subjects was taking ChEIs. Approval was obtained from the Local Ethics committee. Informed consent was obtained from all subjects before participation in the study.

2.2. Afferent inhibition by somatosensory input from the median nerve

Conditioning stimuli were delivered through bar electrodes placed at the median nerve at the wrist (cathode proximal) with electrical stimuli of 0.2 ms duration. The intensity of the conditioning stimulus was set at just above the level for evoking a visible thumb twitch. As the test cortical stimulation, TMS of the left motor cortex was applied to the contralateral scalp with a high-power Magstim 200 magnetic stimulator (Magstim Co., Dyfed, Wales). The center of the linear contiguous segment of a figure-of-eight coil, with each lobe 8 cm in diameter (Magstim Co., Dyfed, Wales), was placed over a point 5 cm lateral to the vertex on the interaural line. The coil was angled 45° to the parasagittal plane so that current in the center of the coil flowed in an anteromedial-to-posterolateral direction (Mills et al., 1992). The resting motor threshold (RMT) was defined as the lowest stimulator output intensity capable of inducing MEPs of at least 50 μV peak-to-peak amplitude in the target muscle (Rossini et al., 1994). The intensity of the test stimulation was set to evoke a muscle response with a peak-to-peak amplitude of approximately 1 mV. Electromyography (EMG) from the right first dorsal interosseous (FDI) muscle was carried out using paired Ag–AgCl disk electrodes. Signals were amplified with a band pass of 5 Hz–10 kHz. The interstimulus intervals (ISIs) between the conditioning stimuli and the test stimuli were set at 20, 40, 100, 200 and 600 ms. Each ISI was chosen in random order by a computer program (Spike 2 ver.4; Cambridge Electronic Design, Cambridge, UK). During the experiment, EMG activity was sampled and stored continuously at a sampling rate of 5 kHz for off-line analysis (micro1401; Cambridge Electronic Design, Cambridge, UK). To determine the MEP, five epochs of EMG for each ISI were averaged. The peak-to-peak amplitude of MEP for each ISI was measured. The MEP amplitude ratio was expressed by the MEP test divided by the MEP unconditioned (TMS alone). According to Di Lazzaro's nomenclature, an inhibitory effect that occurred at ISIs as short as 20 ms was defined as SAI (Di Lazzaro et al., 2002).

Table 1
Characteristics of subjects

	AD ($n = 12$)	MCI ($n = 16$)	NC ($n = 15$)
Resting motor threshold (%)	46.83 ± 8.11	48.06 ± 12.55	53.00 ± 9.60
SAI	0.86 ± 0.35*	0.39 ± 0.26	0.41 ± 0.26
MMSE	21.8 ± 2.8†	27.0 ± 3.8	

Mean ± SD, *Two-way ANOVA; $P < 0.001$ vs. NC, †one-way ANOVA; $p < 0.05$ vs. MCI.

2.3. Statistical analyses

We used one-way analysis of variance (ANOVA) to compare age and Mini-Mental State Examination (MMSE) scores. Two-way ANOVA using within factors of ISIs and diagnoses was employed to compare SAI across groups. Post hoc comparisons were made using Bonferroni's method. A value of $P < 0.05$ was used as the significance threshold.

3. Results

The MMSE scores for each group are shown in Table 1. Post hoc comparisons revealed that the score differed sig-

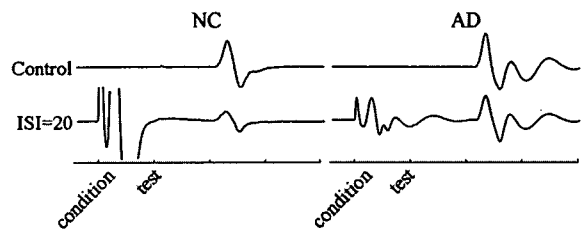

Fig. 1. Raw data traces showing SAI by somatosensory input from the hand in an NC subject and in an AD subject. The top traces show the averaged response by TMS alone. The low traces show the averaged response when TMS was conditioned by a median nerve stimulation with an ISI of 20 ms. In the NC subject, the median nerve conditioning stimulus suppressed the MEP (44% of control), while in the AD subject, the MEP was not very inhibited by conditioning stimulus (83% of control).

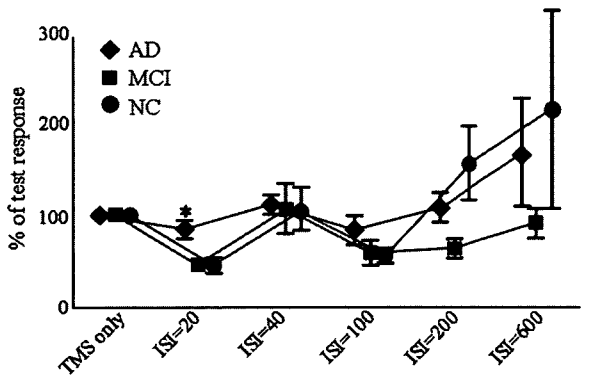

Fig. 2. Afferent inhibition by somatosensory input from the median nerve. Ratios of mean conditioned/unconditioned MEP amplitude are plotted. Conditioning median nerve stimulation preceded TMS of the contralateral motor cortex by five different ISIs (20, 40, 100, 200 and 600 ms). Error bars indicate 1 standard error. Clear short (ISI = 20 ms) and long (ISI = 100 ms) afferent inhibitions were observed in normal controls. SAI was significantly reduced in AD subjects (86%), whereas MCI subjects had almost the same level compared with NC subjects (39% vs. 41%). The asterisk indicates a significant difference compared with NC subjects ($P < 0.05$).

nificantly across groups [$F(2,43) = 38.52$, $P < 0.05$]. The score was significantly lower (indicating worse functioning) in patients with AD and MCI than in NC subjects (Table 1). Though the RMT tended to be lower in patients with AD and MCI than in NC subjects, there was no significant difference across groups [$F(2,43) = 1.91$, $P = 0.16$]. SAI values (ISI = 20 ms), which are the mean MEP amplitude ratio compared with unconditioned response, are shown in Table 1. The SAI values were 0.41 ± 0.26, 0.39 ± 0.26 and 0.86 ± 0.35 in NC, MCI and AD subjects, respectively (Table 1 and Figs. 1 and 2). Two-way ANOVA showed an effect for both diagnosis [$F(2,273) = 3.08$, $P < 0.05$] and ISI [$F(5,270) = 4.34$, $P < 0.005$]. Post hoc comparisons revealed significantly reduced SAI at ISI = 20 ms in AD patients compared with NC subjects and MCI patients ($P < 0.001$) (Table 1 and Figs. 1–3). There was no significant difference between the groups when data for ISI = 20 ms were excluded.

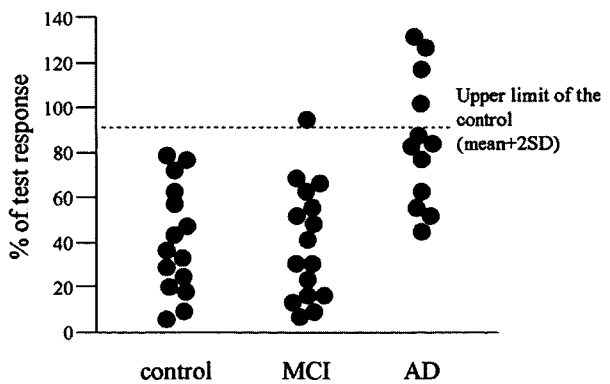

Fig. 3. Scatterplot showing individual values of SAI in patients with MCI ($n = 16$), in patients with AD ($n = 12$) and in control subjects ($n = 15$). The dotted line represents upper limit of the control (mean + 2SD).

4. Discussion

In this study, reduced SAI was observed in AD subjects. This finding is consistent with a previous report (Di Lazzaro et al., 2002). However, we found no reduction of SAI in MCI subjects.

Conditioning stimulation by electrical median nerve shock suppresses the amplitude of MEP evoked by TMS of the contralateral motor cortex with an ISI of 19–21 ms. This phenomenon has been proven to result from reduced cortical excitability followed by sensory input from the hand (Tokimura et al., 2000). Di Lazzaro and colleagues used pharmacological intervention for this phenomenon and found this cortical network was reduced by M1 receptor blockade (Di Lazzaro et al., 2000). Furthermore, they also reported that SAI was reduced in AD patients, and the reduction was recovered by administration of a single oral dose of rivastigmine, a ChEI (Di Lazzaro et al., 2002). SAI is also influenced by benzodiazepines (Di Lazzaro et al., 2005a,b) in normal subjects and dopaminergic drugs in patients with Parkinson's disease (Sailer et al., 2003). Though the reduction in SAI by benzodiazepines may be explained by an inhibition of acetylcholine release, other mechanisms, such as interactions among dopaminergic, GABAergic and acetylcholine neurons, may contribute to the modulation of SAI. While the contribution of neurotransmitters other than acetylcholine in SAI is not fully understood, SAI is useful to probe the integrity of cortical cholinergic neural circuits (Ziemann, 2004).

Recently, clinical and research concerns with MCI have been growing. What is important is the high rate of conversion of MCI to AD. The annual conversion rate for MCI to dementia has been reported to be 10–15% (Morris et al., 2001). This value is significantly higher than that in healthy elderly individuals (1–2% per annum) (Petersen et al., 1999). Even though not all MCI patients convert to AD, MCI is thought to be a prodromal state of AD. An understanding of the pathophysiology of MCI may lead to the discovery of a suitable treatment to prevent the progression to AD.

An important pathophysiological concept of AD is the deficit in cholinergic function. Based on this cholinergic hypothesis, several ChEIs have been developed, have entered the market and, although not curative, have demonstrated benefits in terms of cognition, global function and activities of daily living (Evans et al., 2004). This now raises the question of whether MCI patients already have cholinergic dysfunction, like AD patients. If so, early treatment of MCI patients using ChEIs may have some benefit, such as slowing disease progression and improving quality of life.

Mufson and colleagues reported that a significant reduction in the number of nucleus basalis p75(NTR)-immunoreactive neurons was seen in individuals with MCI (Mufson et al., 2002). Another in vivo study using functional MRI showed that short-term treatment with a ChEI appeared to enhance the activity of the frontal circuitry in patients with MCI (Saykin et al., 2004). Though these data

support the concept that degeneration of the cholinergic basal forebrain occurs early in the disease, some reports showed results contrary to this concept. Gilmor and coworkers showed that the number of choline acetyltransferase (ChAT)-immunoreactive neurons did not decrease in the basal forebrain in patients with MCI (Gilmor et al., 1999). A randomized placebo-controlled trial found that 24 weeks of administration of the ChEI donepezil did not show a significant treatment effect in MCI patients (Salloway et al., 2004). The application of a simple cholinergic hypothesis to MCI should be done carefully, because the status of the cholinergic regulation in MCI is unclear. The present study suggests that cortical cholinergic neural circuits may be normal in MCI patients. SAI reflects the interaction between the sensory and motor systems. This interaction is thought to occur in the cerebral cortex (Tokimura et al., 2000). If the degeneration is localized in the nucleus basalis of Meynert, or if the cortical cholinergic deficiency is compensated by feed-back control, the degenerative process may not be reflected by SAI. The elevation of ChAT activity in the superior frontal cortex and hippocampus in MCI subjects is evidence that some compensatory responses exist during the early stage of dementia (DeKosky et al., 2002). In this study, we found this to be the case in an in vivo neurophysiological experiment. A neuropsychological experiment showed reduced hippocampal function in untreated MCI patients, and administration of galantamine, a ChEI, improved hippocampal function. Taken together, these results suggest that some imbalance in the state of the cholinergic system between the hippocampus and the neocortex may exist. As the disease progresses, the imbalance may gradually acquire an equilibrium. Finally, the abnormal cortical cholinergic circuit estimated by the SAI in AD becomes apparent. Though not all MCI cases convert to AD, the critical point at which SAI becomes abnormal will be able to be captured by this simple method. In fact, one of MCI patients exceeded mean + 2SD of SAI value (Fig. 3). Long-term studies in these patients are necessary to clarify the different state of the cholinergic system between MCI and AD.

Acknowledgements

This study was supported by a Grant-in-Aid from the Research Committee on rTMS treatment of movement disorders, the Ministry of Health and Welfare of Japan (17231401).

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 doi:10.1111/j.1468-1331.2007.01931.x

Autoantibodies against M1 muscarinic acetylcholine receptor in myasthenic disorders

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Keywords:
autoimmunity, calcium channel, Lambert–Eaton myasthenic syndrome, muscarinic acetylcholine receptor, myasthenia gravis

Received 14 May 2007
Accepted 2 August 2007

The Lambert–Eaton myasthenic syndrome (LEMS), often associated with small-cell lung carcinoma (SCLC), is a disorder of acetylcholine (ACh) release from motor nerve terminals. In most patients, it is caused by autoantibodies against the P/Q-type voltage-gated calcium channels (VGCC) that trigger ACh release. However, these antibodies are not detected in approximately 15% of clinically and electrophysiologically typical cases. The M1-type pre-synaptic muscarinic ACh receptor (M1 mAChR) modulates cholinergic neuromuscular transmission by linking to P/Q-type VGCC, and may partially compensate for the reduced calcium entry. Immunoblotting against solubilized human M1 mAChR, we detected autoantibodies in: (a) 14 of 20 (70%) anti-VGCC-positive LEMS patients; (b) all five anti-VGCC-negative LEMS patients, one of whose serum had previously passively transferred LEMS-type electrophysiological defects to mice; (c) all five LEMS patients with autonomic symptoms; (d) seven of 25 (28%) myasthenia gravis (MG) patients in whom increased ACh release partially compensates for post-synaptic defects; (e) none of 10 SCLC patients without LEMS. Although not proving primary pathogenicity of anti-M1 mAChR antibodies, the present results highlight their potential to affect synaptic compensatory mechanisms, more in LEMS than MG.

Introduction

The neuromuscular transmission defect in the Lambert–Eaton myasthenic syndrome (LEMS) is caused by reduced quantal acetylcholine (ACh) release which is caused mainly by autoantibodies against P/Q-type voltage-gated calcium channels (VGCC) [1]. These antibodies are not found in about 15% of patients with clinically typical LEMS [2,3]. As previously reported [3], their serum immunoglobulin G (IgG) can nonetheless passively transfer electrophysiological LEMS features to mice, just like the IgGs from 'anti-VGCC-positive' LEMS [3–5]. Either the present assay misses some antibodies specific for P/Q-type VGCCs at the nerve terminal or these patients have other autoantibodies against distinct targets. Synaptotagmin acts as an exocytotic calcium receptor in the nerve terminal, and is partially exposed at the surface of the nerve terminal during exocytosis [1]. This pre-synaptic protein has been studied as a candidate target involved in the etiology of LEMS [6,7]. Although active immunization caused an animal model of LEMS [8], we detected anti-synaptotagmin I antibodies only in a proportion of LEMS patients [9]. Hoping to identify targets other than the P/Q-type VGCC, we tested LEMS patients for antibodies to other nerve terminal antigens, focusing on candidates involved in compensating for defects in neuromuscular transmission. In myasthenia gravis (MG), ACh release from the nerve terminal is upregulated when synaptic transmission is post-synaptically impaired by anti-nicotinic ACh receptor (AChR) antibodies [10,11]. This compensatory mechanism is suggested to act indirectly via pre-synaptic (intra-terminal) signaling pathways [10].

In view of these reports, we focused on M1-type pre-synaptic muscarinic AChR (M1 mAChR), because it is expressed extracellularly at the neuromuscular junction where it modulates cholinergic neuromuscular transmission [12]. It links to the P/Q-type VGCC, eventually promoting calcium-dependent ACh release [13]. In the present study, therefore, we tested sera from patients with LEMS and MG against pre-synaptic M1 mAChR, using, as disease controls, serum samples from patients without LEMS but with small-cell lung carcinoma (SCLC, found in about 60% of LEMS patients [3,14]).

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Patients and methods

Patients

Serum samples from 25 patients with clinically and electrophysiologically typical features of LEMS [3,14,15] were studied; as Table 1 summarizes, 15 patients had SCLC and five patients had autonomic symptoms. Antibodies to the P/Q-type VGCC were tested in immunoprecipitation assays using ^{125}I-ω-conotoxin MVIIC-labeled human cerebellar extract (control, < 20 pmol/l) [7]. Serological studies were also performed in 25 generalized MG patients (seven men and 18 women, aged from 20 to 60 years) positive for anti-nicotinic muscle AChR antibodies (5.6–120 nmol/l; control, < 0.1 nmol/l), 17 of whom had thymomas. As disease controls, we tested 10 patients with histologically confirmed SCLC without LEMS and negative for anti-P/Q-type VGCC antibodies. Control serum samples were obtained from 30 healthy volunteers (20 men and 10 women, aged 22–59 years). All samples were taken with informed consent and Ethics Committee approval.

Immunoblot analysis of anti-M1 mAChR antibodies

Cloned Chinese hamster ovary (CHO) cells stably expressing human M1 mAChR, and non-transfected CHO (control) cells [16], were incubated at 37°C in a humidified atmosphere (5% CO_2) as monolayer cultures in Ham's F12 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Moregate Exports, Bulimba, Australia). Cells were grown to confluence, washed with phosphate-buffered saline (PBS), and scraped into ice-cold PBS. Cell-membrane proteins were prepared using Qproteome

Table 1 Clinical and immunological profiles of 25 patients with Lambert-Eaton myasthenic syndrome

		Muscle action potentials[a]				Antibodies to:	
No. patients	Gender/onset age (years)	Single at rest (mV)	Potentiation at 50 Hz (%)	SCLC[b]	Autonomic symptoms[c]	P/Q-type VGCC[d] (pmol/l)	M1 mAChR[e] (immunoblots)
1	M/67	1.0	740	No	No	8235	–
2	M/68	0.4	804	Yes	Yes	6303	+
3	M/74	0.5	570	No	Yes	3805	+
4	M/63	1.5	233	No	No	2068	+
5	M/68	2.5	480	Yes	No	1814	–
6	M/55	1.0	133	Yes	No	1481	+
7	M/64	1.8	500	Yes	No	1275	+
8	M/67	0.5	700	Yes	No	1216	+
9	M/68	1.4	484	Yes	No	381	+
10	M/58	0.4	340	No	No	343	+
11	M/47	2.7	200	Yes	No	342	–
12	M/57	0.3	979	Yes	No	256	–
13	M/41	1.5	200	Yes	No	236	+
14	M/62	1.0	600	Yes	No	216	+
15	M/68	1.7	290	Yes	No	148	–
16	M/74	3.0	242	Yes	No	138	+
17	M/62	1.3	670	No	No	136	+
18	M/58	2.1	122	Yes	No	124	–
19	M/69	0.1	220	No	No	49	+
20	M/68	3.1	200	Yes	No	43	+
21	F/55	0.8	370	No	Yes	< 1.0	+
22	M/63	1.8	267	No	No	< 1.0	+
23	F/63	0.8	541	No	Yes	< 1.0	+
24	M/68	0.7	200	No	Yes	< 1.0	+
25[f]	M/79	2.6	257	Yes	No	< 1.0	+

[a]Recording by surface electrodes placed on the thenar muscle with supramaximal stimulation to the median nerve, post-tetanic potentiation of muscle action potential (MAP) is the single MAP amplitude recorded immediately after 50 Hz stimulation for 3 s, expressed as the percentage of that at rest (control: 5–10.8 mV at rest and less than 110% after 50 Hz).

[b]SCLC, small-cell lung carcinoma.

[c]Autonomic symptoms include dry mouth, constipation and impaired sweating.

[d]VGCC, voltage-gated calcium channel (control for antibodies, < 20 pmol/l).

[e]M1 mAChR, M1-type of muscarinic acetylcholine receptor [positive (+) and negative (–) for antibodies].

[f]IgG from this patient's serum transferred the electrophysiological features of Lambert–Eaton myasthenic syndrome to mice.

Cell Compartment Kit (Qiagen Inc., Valencia, CA, USA). Their biological activity was confirmed by Scatchard plots for specific [^{3}H]*N*-methylscopolamine binding and its displacement by competitive drugs from membranes prepared from the M1 mAChR transfectants [16].

M1 mAChR or control cell membrane fractions (7.5 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NuPAGE 4–12% Bis-Tris Gel) (Invitrogen, Carlsbad, CA, USA), transferred to Immune-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA) and incubated with either patient sera (1:100 dilution in 1% skimmed milk; Nakarai Teq, Kyoto, Japan) in Tris-buffered saline/0.05% Tween-20 (dilution buffer) or rabbit anti-human M1 mAChR polyclonal antibody (AB5164, raised against a fusion protein including residues 227–353 human M1 mAChR; Chemicon International, Inc., Temecula, CA, USA) (1:200 dilution in dilution buffer) for 2 h at 22°C. Membranes were then incubated with horseradish peroxidase-conjugated anti-human (or anti-rabbit) IgG (1:500 in dilution buffer) for 1 h at 22°C. Bound antibodies were detected using 4-chloro-naphthol reagent (PerkinElmer Life Science, Boston, MA, USA).

Results

In immunoblots of membrane fractions from human M1 mAChR-transfected CHO cells, serum samples from 14 of 20 (70%) LEMS patients positive for anti-P/Q-type VGCC antibodies and all five who were negative labeled a 51-kDa band (a in lanes 2–4; Fig. 1) at the same migration position as that visualized by the rabbit polyclonal antibody raised against human M1 mAChR (a in lane 1; Fig. 1). No staining was seen in parallel immunoblots with the membrane fraction from non-transfected CHO cells (b in lanes 1–7; Fig. 1) (Table 1).

Of the 20 anti-VGCC-positive LEMS patients, nine of the 14 with SCLC, including two with autonomic symptoms, were positive for anti-M1 mAChR antibodies (a in lane 2; Fig. 1; no. 2 in Table 1). So were all

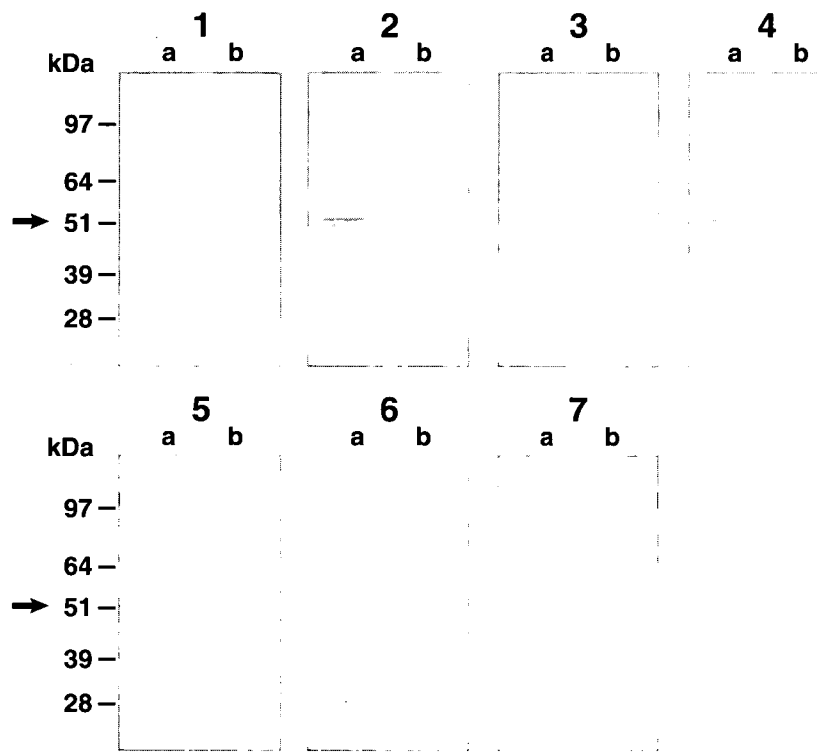

Figure 1 Immunoblots of the human M1 mAChR expressed in CHO cells are shown with the anti-human M1 mAChR polyclonal rabbit antibody (lane 1), serum samples from patients (lanes 2–6) and a control serum (lane 7). For each sample, lane 'a' represents the M1 mAChR-expressing and lane 'b' the non-transfected CHO cells. Lane 2: from an anti-P/Q-type VGCC-positive LEMS patient (no. 2 in Table 1); lane 3: from an anti-VGCC-negative LEMS patient whose serum IgG transferred the electrophysiological features of LEMS to mice (no. 25 in Table 1); lane 4: from an anti-P/Q-type VGCC-negative LEMS patient with autonomic symptoms (no. 21 in Table 1); lane 5: from an MG patient; lane 6: from an SCLC patient without LEMS. Arrows indicate the reactivity of sera with a 51-kDa band migrating in the same position as the human M1 mAChR detected by the specific polyclonal antibody. Molecular mass markers in kDa are shown on the left.

five anti-VGCC-negative LEMS sera, including sample no. 25 (Table 1) which had previously passively transferred electrophysiological LEMS features to mice [3] and was previously confirmed to be negative for anti-synaptotagmin I in immunoblot (data not shown) (a in lane 3; Fig. 1); three of these patients also had autonomic symptoms (a in lane 4; Fig. 1 – no. 21, 23 and 24 in Table 1). The serum samples from 10 patients with SCLC without LEMS showed no reactivity with the human M1 mAChR (a in lane 6; Fig. 1), nor did the 30 control serum samples (a in lane 7; Fig. 1). Notably, of the 25 MG sera, seven (28%) (four with thymomas) also reacted with the human M1 mAChR as shown by a 51-kDa band (a in lane 5; Fig. 1).

Discussion

We report, for the first time, autoantibodies against M1 mAChR in 70% of LEMS patients with anti-VGCC antibodies and 100% of those without, but in none of 10 SCLC patients without neurological signs. However, these antibodies were also present in some patients with MG, even though their electrophysiological defects are very different from those in the LEMS. While the primary pathogenicity of the anti-M1 mAChR antibodies is not yet clear, they may nevertheless hold clues to other potential targets that could be involved in synaptic compensatory mechanisms in the myasthenic disorders.

Modulators of ACh release: potential targets for pathogenic autoantibodies

The G-protein-coupled mAChRs modulate ACh release in cholinergic synapses at the adult skeletal neuromuscular junction [12,13]. When neuromuscular transmission is defective, the balance between M1-enhancers and M2-inhibitors (via intra-cellular serine–threonine kinase-mediated transduction pathways) [12,13] is shifted to the M1 type [12,13,17] – which is expressed in the nerve terminal [12] and partly exposed extracellularly [18]. By contrast, the M2 mAChR may not be accessible to autoantibodies because it is expressed intra-terminally in the adult neuromuscular junction [12]. The M3 mAChR is expressed in the pre-terminal axons but is not coupled to ACh release modulation at the neuromuscular junction [12].

The 51-kDa band identified by patients' sera in the present immunoblot analysis against human M1 mAChR appears identical to that reported in neuronal PC12D cells where M1 mAChR activates phospholipase C (PLC) [19]. Stimulation of M1 mAChR leads to activation of PLC and then of protein kinase C (PKC) [13], that generates diacylglycerol from phosphatidylinositol-4,5-bisphosphate [19], which, in turn, enhances P/Q-type VGCC-mediated ACh release [13]. We therefore propose that the M1 mAChR is implicated in pre-synaptic compensation for the impaired calcium entry through VGCC, and that the autoantibodies we report here may impair that compensation, especially in LEMS. Other potential compensatory mechanisms could include a shift in dependence of ACh release from the P/Q-type to other types of VGCC such as L-, N- and/or R-types [5,20], and a switch from slow- to fast-mode synaptic vesicle recycling [21,22]. The anti-M1 mAChR antibodies we detected did not correlate clearly with an electrophysiological severity under the condition that the data were obtained from the hand muscle (Table 1), as suspected by the possibility that the electrophysiology of single muscle may not accurately reflect overall severity.

The prevalence in anti-M1 mAChR antibodies in the present study may hold clues to the pathophysiology in anti-VGCC-negative LEMS patients. Pharmacologically, M1 mAChRs are apparently involved in normal synaptic function; physiological ACh quantal release is reduced by the specific M1 mAChR blocker (pirenzepine) in 'cut muscle preparations', i.e. at standard ion concentrations and without added drugs [13]. Immunologically, serum IgG from our anti-VGCC-negative/anti-M1 mAChR-positive patient (no. 25 in Table 1) transferred electrophysiological features of LEMS to mice [3], the neuromuscular junction of which expresses pre-synaptic M1 mAChRs accessible to extracellularly applied agents [17] including any transferred autoantibodies. In addition to these transfer and pharmacological experiments, to test the pathogenicity of the anti-M1 mAChR autoantibodies requires further studies in cultured cell lines and *in vivo* in experimental animals to assess their effects on mAChRs at the neuromuscular synapse. As mAChR-deficient mice reportedly show physiological, pharmacological and biochemical deficits in the central and peripheral nervous systems [23], the behavior of their neuromuscular synapses may help us understand mAChR-dependent compensatory mechanisms and their involvement in myasthenic disorders.

While mAChRs are expressed by SCLC cell lines [24], autoantibodies against them were equally prevalent in the LEMS patients with and without SCLC (Table 1); as with the anti-VGCC antibodies, there must be distinct stimuli in the patients without tumors.

Autonomic effects in the LEMS

Autonomic symptoms are frequently reported in Caucasian LEMS patients [14] and attributed to anti-VGCC antibodies [25]. Interestingly, they were noted in

only five of the 25 Japanese LEMS patients in the present study, as in our previous survey (37% of 110 Japanese LEMS patients) [3]. However, some who had an abnormal Saxon test (a quantitative test for salivary secretion) did not complain of dry mouth; i.e. they were asymptomatic [3], so we suspected that autonomic symptoms are under-reported in Japanese LEMS. Their apparently higher prevalence in three of five anti-VGCC-negative/anti-M1 mAChR-positive LEMS patients demands confirmation in a larger series of these unusual patients. Similar to the M3 mAChR, the M1 mAChR mediates cholinergic stimulation of glandular secretion [26]. The present study does not include any Sjögren syndrome patients whose serum samples reportedly contain anti-M1 and anti-M3 mAChR antibodies [27].

Anti-M1 mAChR autoantibodies in MG

Antibodies against M1 mAChR were detected in only seven of the 25 MG patients (28%). In the others, these receptors should still be available to compensate by upregulating ACh quantal release [10,11] via pre-synaptic calcium influx mechanisms such as those proposed here. Additional compensatory mechanisms in MG could include upregulation of pre-synaptic proteins such as α-neurexins [28] and/or of the AChR itself/the protein(s) essential for its clustering [29,30].

Acknowledgements

We are grateful to Drs Bethan Lang and Nick Willcox (Neurosciences Group, Weatherall Institute for Molecular Medicine, University of Oxford, Oxford, UK) for their thoughtful advice on the study and critical review of the manuscript.

Conflict of interests

The authors have no conflicts of interest to declare.

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Movement Disorders
Vol. 22, No. 6, 2007, pp. 857–862

Clinical and Genetic Characterizations of 16q-Linked Autosomal Dominant Spinocerebellar Ataxia (AD-SCA) and Frequency Analysis of AD-SCA in the Japanese Population

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Abstract: Autosomal dominant spinocerebellar ataxias (AD-SCAs) form a clinically and genetically heterogeneous group of neurodegenerative disorders. Recently, a single nucleotide substitution in the 5′-untranslated region of the *puratrophin-1* gene was found to be associated with one type of AD-SCA linked to chromosome 16q (16q-SCA). To obtain further insight into the contribution of the C-to-T substitution in the *puratrophin-1* gene to the clinical and genetic characteristics of patients with 16q-SCA, we analyzed 686 families with 719 individuals diagnosed with progressive ataxia. We found C-to-T substitution in the *puratrophin-1* gene in 57 unrelated families with 65 affected individuals. The mean age at onset in the patients with 16q-SCA was 59.1 (range, 46–77). Ataxia is the most common initial symptom. The elderly patients over 65 occasionally showed other accompanying clinical features including abnormalities in tendon reflexes, involuntary movements, and reduced vibration sense. We also examined the frequency of the AD-SCA subtype, considering the effects of age at onset. In the 686 AD-SCA families, SCA6 and Machado-Joseph disease/SCA3 are frequent subtypes, followed by dentatorubral-pallidoluysian atrophy and 16q-SCA. 16q-SCA is not a rare subtype of Japanese AD-SCA, particularly in patients with ages at onset over 60. © 2007 Movement Disorder Society

Key words: autosomal dominant cerebellar ataxia; 16q-SCA; genetic testing; common haplotype; frequency analysis.

Autosomal dominant spinocerebellar ataxias (AD-SCAs) form a clinically and genetically heterogeneous group of neurodegenerative disorders, characterized by progressive cerebellar signs and symptoms.[1,2] Recent advances in molecular genetics have elucidated at least 13 genes and 15 additional loci responsible for AD-SCA.[3,4] The mutational basis of most causative genes for AD-SCAs is a CAG repeat expansion in the coding region of the genes for SCA1, SCA2, Machado-Joseph disease (MJD)/SCA3, SCA6, SCA7, SCA17, and dentatorubal-pallidolyusian atrophy (DRPLA).[3,4] Although SCA8 was initially proposed to be caused by a CTG repeat expansions in the 3′-untranslated region (UTR) of the *SCA8* gene,[5] it remains controversial whether the CTG repeat expansion is casual or only polymorphisms. In SCA12, a CAG repeat expansion in the 5′ region of

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Received 2 December 2006; Revised 31 December 2006; Accepted 29 January 2007
Published online 13 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/mds.21443

the *PPP2R2B* gene, which does not encode a polyglutamine tract, was found in a German family.[6] In addition to mutations with repeat expansions, mutations resulting in the change in amino acid composition of the protein encoded by the fibroblast growth factor 14 gene for SCA27,[7] the protein kinase C γ gene for SCA14,[8] the voltage-gated potassium channel, KCNC3, for SCA12,[9] and β-III spectrin for SCA5[10] have been reported to cause AD-SCA in a few families. The detection of these mutations has enabled the classification of AD-SCAs by molecular analysis.

SCA4, originally described in a large pedigree of Scandinavian origin in Utah, is characterized by progressive cerebellar ataxia with sensory axonal neuropathy, and was mapped to 16q22.1.[11] Another SCA4 patient with a similar clinical phenotype was subsequently reported in a German family.[12] Although Japanese AD-SCA families were also mapped to the same loci of SCA4 and termed 16q-linked SCA,[13] 16q-SCA is clinically distinct from the SCA4 in the Utah and German families. Clinical presentations of 16q-SCA are characterized by pure cerebellar ataxia, without signs of peripheral neuropathy. In addition, the ages at onset of patients with 16q-SCA are later than those reported in SCA4 patients. Recently, a single-nucleotide substitution (C-to-G), in 5′ UTR of the *puratrophin-1* gene, was identified in patients with 16q-SCA.[14] These families share a single common haplotype between D16S421 and CATG003 at 16q22.1.[14] Although microscopic aggregates in the cytoplasm of Purkinje cells in brains of the 16q-SCA patients are strongly stained by the puratrophin-1 antibody,[14] the pathological relevance of the altered anti-puratrophin-1 immunoreactivity in the cerebellum remains unclear.

Here, we report detailed analyses of clinical and genetical characteristics of 16q-SCA patients with the single-nucleotide substitution of the *puratrophin-1* gene by screening a large data set of AD-SCA patients. Furthermore, we present a frequency analysis using one of the largest cohorts of 686 AD-SCA pedigrees on the basis of genetic testing.

PATIENTS AND METHODS

Patients

In our study, 686 unrelated families with 719 affected individuals presenting progressive ataxia as a cardinal clinical feature, who were referred to our institute (Brain Research Institute, Niigata University) on a consecutive basis from January 1995 to December 2005, were enrolled. Pedigrees were considered to have a dominant SCA when affected individuals with progressive ataxia were observed in at least two generations. Sporadic cases with ataxia were not included in this study. Geographically, collected families are residents in Honshu Island, which is the main island of Japan. Age at onset and family history were determined on the basis of historical information provided by the patients or their close relatives. All the patients who enrolled in the study gave their written informed consent before molecular genetic investigations.

Molecular Studies

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures. Screening for CAG repeat expansion of SCA1, SCA2, SCA3/MJD, SCA6, SCA7, SCA17, and DRPLA was performed as described previously.[15,16] In this study, SCA10 and SCA12 expansions were not analyzed. The C-to-T substitution in the 5′ UTR of the *puratrophin-1* gene was analyzed by PCR-RFLP using EcoNI, as described previously.[14] The nucleotide substitution was further analyzed by direct sequencing using a standard procedure using a BigDye terminator on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems, Foster City, CA).

For 16q-SCA families in which at least two patients were available for genetic analysis, we determined the genotype of the patients in the region tightly linked to 16q-SCA using markers on 16q22.1, which include D16S3086, GATA01, D16S421, TA001, GA001, and 17msm. Presumed haplotypes were constructed assuming the least number of recombinations.

RESULTS

C-to-T Substitution in *puratophin-1* Gene and Haplotype Analysis of 16q-SCA

Among 686 AD-SCA families in our cohort, 57 families were identified to have 65 affected individuals, who carried the C-to-T substitution of the *puratrophin-1* gene. Of the 65 affected individuals, 34 were men and 31 were women. We were able to determine presumed haplotype cosegregating with the C-to-T substitution in the *puratrophin-1* gene in the families, in which more than one affected individual were available for genetic analysis. The pedigrees (Peds 1154, 1440, 1432, 2498, and 2946) shared a common genotype of 183–157-213-T-142–200-191 at D16S3086-GATA01-D16S421-*puratrophin-1*-TA001-GA001–17msm (Table 1).

Clinical Features of 16q-SCA Having C-to-T Substitution of *puatrophin-1* Gene

The mean age at onset of the 65 affected individuals with 16q-SCA was 59.1 (range, 46–77 years). This mean

TABLE 1. *Haplotype analysis in families with 16q-SCA*

	D16S3086	GATA01	D16S421	*puratrophin-1*	TA001	GA001	17msm
Ped 1154							
II-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	185	161	213	C	157	206	193
II-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	185	161	213	C	157	206	193
Ped 1432							
I-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	187	149	213	C	152	206	191
I-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	185	153	213	C	152	206	195
II-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	187	149	213	C	152	206	191
Ped 1440							
I-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	157	213	C	140	202	197
I-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	157	213	C	142	203	195
Ped 2498							
I-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	161	213	C	142	202	195
I-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	161	213	C	142	202	195
Ped 2946							
I-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	157	215	C	154	202	197
I-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	183	157	213	C	156	206	193
Ped 3946							
I-1	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	185	157	213	C	150	206	195
I-2	**183**	**157**	**213**	**T**	**142**	**200**	**191**
	185	157	213	C	156	206	195
Ped 2333							
II-3	185	**157**	**213**	C	151	206	193
	185	153	221	C	155	206	195
III-1	183	161	**213**	**T**	**142**	**200**	**191**
	185	161	213	C	156	208	195

Numbers in bold indicate the shared genotype patients in the pedigrees.

age at onset of the 16q-SCA patients is much later than those of other AD-SCAs, including SCA1 (mean age at onset, 40.9 years; range, 16–65 years, n = 22), SCA2 (36.1, 19–60, n = 29), MJD/SCA3 (39.5, 7–75, n = 221), SCA6 (52.5, 25–76, n = 209), SCA17 (37.0, 27–47, n = 2), and DRPLA (30.4, 0–71, n = 170) in our cohort. The clinical characteristics of 16q-SCA patients in our cohort are summarized in Table 2. The initial clinical features were gait ataxia (70%) or dysarthria (28%). On neurological examination, the affected individuals commonly exhibited limb and truncal ataxia (100%) and dysarthria (94%), which were invariably the most prominent features throughout the disease course. Other accompanying clinical features were less frequent, which include gaze-evoked nystagmus (41%), hyperactive (33%) and reduced (11%) muscle stretch reflex, decreased vibration sense (10%), involuntary movements (8%), and dementia (5%). These extracerebellar features

TABLE 2. *Clinical characteristics of patients with 16q-SCA*

Patients examined (n)	65
Men/women (n)	34/31
Age at onset (yr)	59.1 ± 6.9 (range, 46–77)
Age at examination (yr)	67.3 ± 8.4 (range, 47–94)
Duration (yr)	8.2 ± 6.1 (range, 1–23)
Initial symptoms (%)	
Unsteadiness of gait	70
Dysarthria	28
Clinical characteristics (%)	
Limb and truncal ataxia	100
Dysarthria	94
Gaze nystagmus	41
Muscle stretch reflex	
Hyperactive	33
Reduced	11
Reduced vibration sense	10
Involuntary movements	8
Dementia	5

were observed more frequently in elderly patients aged over 65 years. Hearing impairments were inconspicuous in our cohort.

Family in Which C-to-T Substitution in *puratrophin-1* Gene Was Not Cosegregated

We found a unique case (II-3) in Ped 2333, who lacks the C-to-T substitution of the *puratrophin-1* gene; another affected member (III-1) was found to carry this substitution. The patient lacking the substitution developed gait disturbance at age 59, and cerebellar symptoms gradually progressed. Brain MRI at age 69 revealed atrophy of cerebellar hemisphere without brainstem involvement. Both clinical and radiological findings of the patient (II-3) were indistinguishable from those of other 16q-SCA patients. On genotype analysis using markers on 16q22.1, the patient (II-3) without the substitution carried only a limited common haplotype at GATA01 and D16S421, whereas the patient (III-1) with the substitution carried the common haplotype of 16q-SCA between D16S421 and 17msm (Table 1).

Coexistence of C-to-T Substitution in *puratrophin-1* Gene and SCA1 Expansion in Patients

In the genetic screening of AD-SCA, we identified a patient who carried both a CAG repeat expansion (44 repeats) in the *SCA1* gene and 16q-SCA mutations, indicating that this patient has two different types of genetic mutation causing cerebellar dysfunction. He developed mild dysarthria and swallowing disturbance at age 57, followed by unsteadiness of gait at age 58. On neurological examination at age 63, the patient exhibited dysarthia, dysphagia, limb and truncal ataxia, and hyperreflexia. MRI of the patient showed mild atrophy of cerebellar vermis and hemisphere, whereas the brainstem appears to be preserved. He now remains well ambulatory 6 years after the onset.

Frequency Analysis of AD-SCA in Japanese Families

The relative frequenies of AD-SCAs in our cohort are summarized in Figure 1A. From the family-based frequency analysis, SCA6 was found to be the most frequent (28%), followed by MJD/SCA3 (27%), DRPLA (20%), 16q-SCA (8.3%), SCA2 (3.5%), and SCA1 (2.8%). The patient-based frequency analysis revealed that MJD/SCA3 is the most frequent subtype, probably because MJD/SCA3 affects a larger number of subjects within pedigrees than does SCA6. Genetically undetermined AD-SCDs still remain at 10% (Fig. 1A).

We next examined whether the relative frequency of AD-SCAs varies with age at onset (Fig. 1B). Among the patients with juvenile ages at onset earlier than 20 years, DRPLA was by far the most prevalent (71%), followed by MJD/SCA3 (16%). In the patients with early-adult ages at onset ranging from 20 to 39 years, MJD/SCA3 was the most predominant subtype (46%), followed by DRPLA (21%). In the patients with mid-adult ages at onset ranging from 40 to 59 years, SCA6 was the most frequent (35%), followed by MJD/SCA3 (29%). In the patients with late-adult ages at onset over 60 years, SCA6 is the most frequent (46%), followed by 16q-SCA (24%). These results suggest that subtype frequency is considerably variable among groups classified by age at onset.

DISCUSSION

In the present study, we found 57 pedigrees with 65 affected individuals, who carry the C-to-T single-nucleotide substitution in the *puratrophin-1* gene among 686 AD-SCA families. Haplotype analysis revealed that these families with the *puratrophin-1* gene substitution share a common haplotype. These results were consistent with a previous report that showed a strong founder effect in 16q-SCA patients.[14] Recently, Wieczorek et al. reported that neither the C-to-T substitution in 5′-UTR nor mutations in the coding regions of the *puratrophin-1* gene was found in 537 European patients with cerebellar ataxia.[17] Thus, 16q-SCA carrying the C-to-T substitution was exclusively found in Japanese.

The clinical features of the 16q-SCA patients in our study are characterized by late-onset ataxia, which are similar to those reported previously.[13,14,18,19] However, the patients in our cohort showed extracerebellar accompanying symptoms, including nystagmus, hyperactive muscle stretch reflex, or reduced vibration sense, compared with previous reports.[13,19] Since SCA6 is an AD-SCA subtype presenting late-adult onset cerebellar ataxia similarly to 16q-SCA,[20] differential diagnosis between 16q-SCA and SCA6 by clinical presentations is often difficult. Although no single clinical sign can reliably distinguish between these two diseases, there are some differences as follows. The age at onset in the SCA6 patients is younger than that in the 16q-SCA patients. SCA6 patients frequently exhibit gaze-evoked nystagmus (90%),[20] which is less frequent (39%) in 16q-SCA.

In Ped 2333, the proband (III-2) carried the single-nucleotide substitution in the *puratrophin-1* gene, whereas another affected patient (II-3) was found to lack this substitution. However, they presented indistinguishable clinical phenotypes. This finding raises the question of whether this substitution is the actual causative mutation of 16q-SCA. One possible answer lies in the fact that two patients with progressive ataxia in the same family

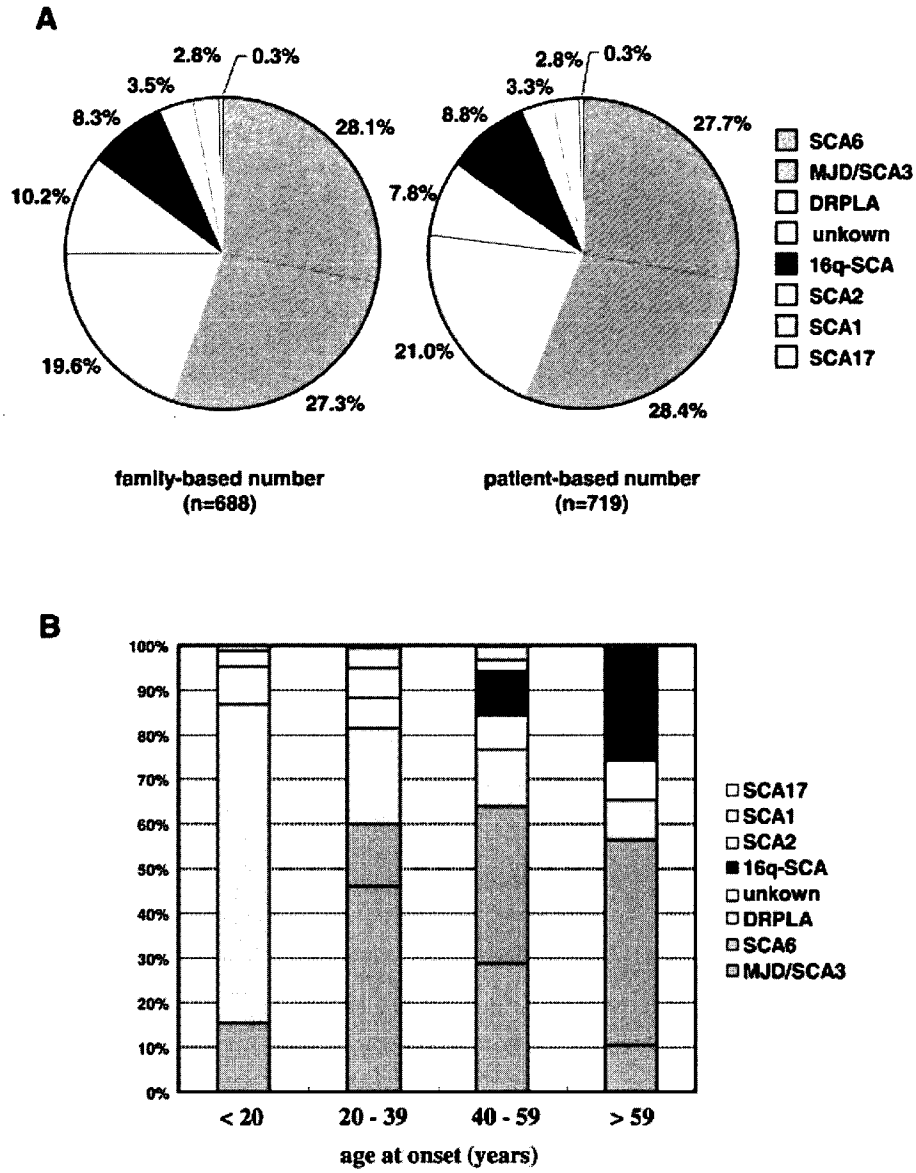

FIG. 1. A. Frequency of SCA subtype in 686 AD-SCA pedigrees determined by family- and patient-based numbers. Each subtype is depicted using different colors, as indicated. Numbers indicate the percentage of each subtype. B. Frequency of AD-SCA subtype among groups, which were classified by age at onset as follows: Group I, under 20 years; Group II, 20 to 39; Group III, 40 to 59; and Group IV, 60 and over.

have suffered from two different cerebellar diseases, and another possible answer is that this substitution is not the causative mutation, but is rather tightly linked to the causative mutation in the surrounding region. Ohata et al. have recently reported the case of a similar 16q-SCA family in which one affected individual lacked the C-to-T substitution of the *puratrophin-1* gene, whereas the other affected individuals carried this substitution.[21] Taken together, our results and those of others indicate the need to further investigate the pathological relevance of the C-to-T substitution in the *puratrophin-1* gene.

The frequency of dominant SCA subtypes is considerably variable within different ethnic groups. Moreover, the previous report suggested the existence of a large geographical difference in the frequency of AD-SCA subtype even in Japan.[22] In this study, we confirmed previous observations that SCA6, MJD/SCA3, and DRPLA are more frequent subtypes in the Japanese population than in other ethnic populations.[15,22] The high prevalence of the SCA3/MJD mutation in Japan is most likely caused by founder effects in the Japanese population.[23] Our study indicates that ~90% of patients with AD-SCA are found to have known mutations, causing either polyglutamine diseases or 16q-SCA in the Japanese population. We also provided the first evidence showing that the relative frequency of AD-SCA consid-