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Cognitive function and cholinergic transmission in patients with subcortical vascular dementia and microbleeds: a TMS study

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Abstract There has been little investigation on the association between cognitive impairment and the microbleeds (MBs) frequently seen in subcortical vascular dementia (SVaD). One possible mechanism of cognitive decline in individuals with SVaD could be disruption of cholinergic fibers by vascular lesions. Central cholinergic circuits in human brain can be tested non-invasively by means of a transcranial magnetic stimulation (TMS) protocol named short latency afferent inhibition (SAI) of motor cortex. In the present study, we used this test in SvaD patients with and without MBs. SAI was evaluated in 13 SVaD patients with MBs (MB-positive group) and the data were compared with those from a group of 15 SVaD patients without MBs (MB-negative group) and with those from 20 healthy subjects. Moreover, we studied covariation of individual SAI values with the Mini-Mental State Examination (MMSE) total score and subscores. SAI was significantly reduced in the MB-positive group when compared with the MB-negative group and the control subjects. Total MMSE score, "attention and calculation" and "orientation" subscores were significantly lower in the MB-positive group than in the MB-negative group; SAI showed a positive correlation with total MMSE score. Adjustment for age, gender, education, presence of lacunae, severe white matter hyperintensities or severe periventricular hyperintensities did not affect these findings. This study provides novel physiological evidence that MBs have an impact on central cholinergic function that is independent of the extent of associated white matter changes and ischaemic stroke. This finding shows that TMS have potential diagnostic and therapeutic implications. TMS studies may help in evaluating the causes of cognitive impairment in cerebrovascular diseases.

Keywords Microbleeds · Subcortical vascular dementia · Transcranial magnetic stimulation · Short latency afferent inhibition · Mini-Mental State Examination

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Introduction

Small-vessel disease (SVD), particularly of an ischemic nature, is generally accepted as a major risk factor for subcortical vascular dementia (SVaD). With recent advent of T2* gradient-echo magnetic resonance imaging (GE-MRI) many studies demonstrated that SVD can produce not only ischemia, but also microbleeds (MBs) (Koennecke 2006; Viswanathan and Chabriat 2006). Since MBs are a manifestation of pathology affecting cerebral small vessels, they are particularly associated with lacunar stroke, cerebral white matter lesions and hypertension (Kwa et al. 1998; Kato et al. 2002). MBs are characterized histologically by the presence

of hemosiderin around small vessels (Fazekas et al. 1999; Tanaka et al. 1999), and appear as small, dot-like lesions of low signal intensity within the brains of patients with haemorrhagic and ischaemic stroke, patients with hypertension, and a smaller proportion of healthy elderly subjects (Chan et al. 1996; Offenbacher et al. 1996; Kwa et al. 1998; Roob et al. 2000). Increasing attention has been paid to associations between cognitive dysfunction and brain MBs. Central cholinergic mechanisms are believed to modulate cognitive and attentional processes in humans (Kasa et al. 1997; Bartus 2000); impaired central cholinergic neurotransmission has a central role in patients with Alzheimer's disease (AD), whereas cholinergic deficit seems to be less relevant in the pathogenesis of SvaD. Interestingly, in AD brain MBs are of special interest as they may play a crucial role in the pathophysiology of the disease (Cordonnier and van der Flier 2011). However, the potential link between MBs and cholinergic function has not been clearly established.

Central cholinergic circuits of human brain can be tested non-invasively by coupling peripheral nerve stimulation with transcranial magnetic stimulation (TMS) of motor cortex. Muscle responses recorded in hand muscles after TMS of the contralateral motor cortex can be suppressed by electrical stimulation of the median nerve if the time interval between stimulation of median nerve and motor cortex is 2–8 ms longer than the time taken by the peripheral nerve afferent input to reach the cortex (Tokimura et al. 2000). This effect, named short latency afferent inhibition (SAI), is produced by inhibitory interactions within the cerebral cortex (Tokimura et al. 2000; Di Lazzaro et al. 2004a). SAI has been shown in healthy subjects to be sensitive to the blockage of muscarinic acetylcholine receptors (Di Lazzaro et al. 2000) and is impaired in cholinergic forms of dementia, such as AD and dementia with Lewy bodies (Di Lazzaro et al. 2002, 2004b, 2007) while it is normal in non-cholinergic forms of dementia such as fronto-temporal dementia (Di Lazzaro et al. 2006). In previous TMS studies, a reduced SAI was found in patients with vascular dementia but not to the same extent as AD. Nardone et al. (2008) reported that SAI responses in patients with SVaD varied widely, ranging from normal to markedly reduced values. In another TMS study significant SAI abnormalities were disclosed in 3 out of 12 patients with vascular dementia (Di Lazzaro et al. 2008).

The objective of the present study was to investigate central cholinergic circuits in SVaD patients with and without MBs. We evaluated SAI in a group of patients with SvaD and MBs and compared the data with those from a group of SVaD patients without MBs and a control group of age-matched healthy individuals. Furthermore, we analyzed correlation between this putative marker of central cholinergic activity, presence of MBs and global cognitive function.

Materials and methods

Patients

We examined 28 patients (17 men and 11 women, mean age 69.5 years, range 57–79 years, 26 right-handed) who met the clinical and imaging criteria of SvaD proposed by Erkinjuntti et al. (2000) and had undergone GE-MRI. MBs were detected in the brain for 13 patients (eight men and five women, mean age 69.7 years, range 58–78 years, 12 right-handed).

Twenty age-matched neurologically healthy controls (13 men and 7 women, mean age 69.2 years, range 55–79 years, 19 right-handed) constituted the control group. None of the controls have MBs.

Exclusion criteria were: inability to undergo cerebral MRI; other concomitant or pre-existing major neurological disease, including epilepsy, presence of major cortical infarcts, evidence of concomitant dementia such as AD, frontotemporal, or reversible dementias; evidence of depression, other psychiatric diseases, drug addiction; systemic diseases or traumatic brain injuries.

All the selected patients were able to understand and carry out the simple tasks required for this electrophysiological study, such as to contract a hand muscle or to keep fully relaxed.

None of the patients were treated with anticholinergic drugs before the study. Administration of all drugs that affect motor cortex excitability in patients and control subjects was discontinued at least 2 weeks before the study.

Patients provided informed consent before participation in this study, which was performed according the Declaration of Helsinki and approved by the institutional Ethics Committee.

Magnetic resonance imaging

MRI was performed using a 1.5-T scanner. MBs were defined on GE-MRI as homogeneous rounded areas of signal loss, with a diameter of 2–10 mm. Two investigators (P.G., S.G.) who were blinded to subject data reviewed the number and location of MBs. Symmetrical hypointensities in the globus pallidum likely to represent calcification and flow void artifacts of pial vessels, as well as hypointense lesions within the subarachnoid space, were disregarded. Moreover, to differentiate MBs from other intra-axial lesions with a hemorrhagic component, only areas of signal loss that were not locally associated with other abnormalities were counted as MBs.

Nine patients displayed only supratentorial MBs and four patients showed both supratentorial and infratentorial MBs. The total number of MBs in the 13 patients was 4,147 and the number of MBs per patient ranged from 4 to 248 with a median number of 17. MBs were most commonly distributed in the cortex and basal ganglia; the cortical MBs were most pronounced in the frontal area. White matter hyperintensities (WMH), periventricular hyperintensities (PVH), and lacunae were independently reviewed by 2 of the authors (F.C., S.G.) who were blinded to subject data. Severity of WHM or PVH on both T2-weighted and fluid-attenuated inversion recovery-weighted images was rated according to the Fazekas scale (WMH: grade 1, punctate; grade 2 early confluence and grade 3 confluent; and PVH: grade 1, caps or lining; grade 2, bands; and grade 3, irregular extension into the deep white matter) (Fazekas et al. 1987).

The lacunar infarction was defined as a focal, small and sharply demarcated lesion, with a diameter of 3–15 mm showing low signal on T1-, high signal on T2-weighted images, and perilesional halo on fluid-attenuated inversion recovery images.

The main demographic characteristics and MRI features of the patients are reported in Table 1.

Neuropsychological examination

Global cognitive function was assessed using the Mini-Mental State Examination (MMSE) (Folstein et al. 1975). MMSE is used worldwide as method to assess cognitive function; this test was chosen in this preliminary study also because it has been used in a recent study that explored the correlation between MBs and cognitive function (Yakushiji et al. 2008).

Transcranial magnetic stimulation

TMS was performed using a High-power Magstim 200 magnetic stimulator (Magstim Co., Whitland, Dyfed, UK) connected to a Bistim module throughout all measurements. A figure-of-eight coil with external loop diameters of 9 cm was held over the motor cortex at the optimum scalp position to elicit motor responses in the first dorsal interosseous (FDI) muscle. The dominant hemisphere was selected for stimulating patients and healthy subjects. The induced current flows in a postero-anterior direction. Motor evoked potentials (MEPs) were recorded via two 9 mm diameter Ag–AgCl electrodes with the active electrode applied over the motor point of the muscle and the reference on the metacarpophalangeal joint of the index finger. MEPs were amplified and filtered (bandwidth 3–3,000 Hz) by D150 amplifiers (Digitimer, Welwyn Garden City, Hertfordshire, UK).

We evaluated the following TMS parameters: the resting motor threshold (RMT), the central motor conduction time (CMCT), the short latency intracortical inhibition (SICI) and intracortical facilitation (ICF) to paired TMS, and the short latency afferent inhibition (SAI).

RMT was defined as the minimum stimulus intensity that produced a liminal motor evoked response (about 50 μV in 50% of 10 trials) at rest. CMCT was calculated by subtracting the peripheral conduction time from spinal cord to muscles from the latency of responses evoked by cortical stimulation with the formula: MEP latency − (F latency + M latency − 1)/2 (Rossini et al. 1994). SICI and ICF were studied using the technique of Kujirai et al. (1993). Two magnetic stimuli were given through the same stimulating coil over the motor cortex and the effect of the first (conditioning) stimulus on the second (test) stimulus was investigated. The intensity of the conditioning stimulus was set to 80% RMT; the second, test, shock intensity was adjusted to evoke a MEP in relaxed FDI with an amplitude of approximately 1 mV, peak-to-peak.

The timing of the conditioning shock was altered in relation to the test shock. Inhibitory interstimulus intervals (ISIs) of 2, 3 and 5 ms and facilitatory ISIs of 7, 10 and 20 ms were investigated. Ten stimuli were delivered at each ISI. For these recordings, muscle relaxation is very important and the subject was given audiovisual feedback at high gain to assist in maintaining complete relaxation. The presentation of conditioned and unconditioned trials was randomized. The amplitude of the conditioned EMG responses was expressed as the percentage of the amplitude of the test EMG responses. The amplitudes of the conditioned responses were averaged obtaining grand mean amplitudes of the three inhibitory and of the three facilitatory ISIs.

SAI was studied using the recently described technique (Tokimura et al. 2000). Conditioning stimuli were single pulses (200 μs) of electrical stimulation (with the cathode positioned proximally) applied through bipolar electrodes to the median nerve at the wrist. The intensity of the conditioning stimuli was set at just over motor threshold for evoking a visible twitch of the thenar muscles. The intensity of the test cortical magnetic shock was adjusted to evoke a muscle response in relaxed FDI with an amplitude of approximately 1 mV peak-to-peak. The conditioning stimulus to the peripheral nerve preceded the test magnetic cortical stimulus. ISIs were determined relative to the latency of the N20 component of the somatosensory evoked potential evoked by stimulation of the median nerve. In the right-handed subjects, the active electrode for recording the N20 potential was attached 3 cm posterior to C3 (10-20 system), and the reference was 3 cm posterior to C4 (vice versa in the left-handed subjects. Five hundred responses were averaged to identify the latency of N20

Table 1 Demographic and MRI parameters of patients with SVaD

Patients	Demographic variables			Neuroradiological measurements		
	Age (years)	Gender	Education (years)	Presence of lacune	Presence of WMH	Presence of PVH
MB-negative group						
1	75	M	8	+	2	1
2	68	M	6	–	2	2
3	57	F	8	–	2	1
4	79	M	5	–	1	0
5	66	F	10	–	2	2
6	60	M	8	+	2	2
7	72	M	8	–	3	1
8	75	M	10	–	1	1
9	64	F	13	+	3	2
10	76	M	5	+	1	1
11	74	F	8	–	2	0
12	72	F	17	+	2	2
13	64	M	8	–	1	0
14	71	M	17	+	1	0
15	66	F	10	–	2	1
MB-positive group						
1	65	F	8	+	1	0
2	75	M	5	–	2	1
3	74	M	8	–	2	2
4	68	M	5	+	3	1
5	78	F	13	+	2	1
6	66	F	8	+	3	2
7	70	M	17	–	1	0
8	58	M	10	–	1	1
9	74	F	9	–	1	0
10	72	M	8	+	2	1
11	71	M	7	+	2	2
12	62	F	15	–	1	2
13	73	M	8	+	2	1

WMH white matter hyperintensities. Grade 1: punctuate; grade 2: early confluence; grade 3: confluent. *PVH* periventricular hyperintensities. Grade 1: caps or lining; grade 2: bands; grade 3: irregular extension into the deep white matter

peak. ISIs from the latency of the N20 component plus 2 ms to the latency of the N20 component plus 8 ms were investigated in steps of 1 ms.

Eight stimuli were delivered at each ISI. We calculated an average of the MEP obtained after cortical magnetic stimulation alone and of the MEP obtained by conditioning cortical magnetic stimulus with a peripheral stimulus to the median nerve at the wrist at the seven different ISIs studied. The amplitude of the conditioned MEP was expressed as percentage of the amplitude of the test MEP. The percentage inhibition of the conditioned responses at the seven different ISIs was averaged to obtain a grand mean. Subjects were given audio-visual feedback at high gain to assist in maintaining complete relaxation.

To clarify a possible spinal or peripheral contribution on the motor cortex excitability parameters, supramaximal stimulation (0.2-ms square-wave constant current pulses) of the ulnar nerve at the wrist was used to assess spinal and peripheral motor excitability. While FDI was relaxed, the peak-to-peak amplitude of F waves (average, 20 trials) and CMAP (maximum, 3 trials) were determined. We identified the F waves according to the criteria reported by the International Federation of Clinical Neurophysiology as responses that are variable in their latency, amplitude and configuration but that which occur grouped with a consistent range of latency (Kimura et al. 1994).

To test if SAI was effectively sensitive to changes in cholinergic activity, RMT and SAI were examined in four

patients of the MB-positive group and in four patients of the MB-negative group before and after the administration of a single dose of 5 mg of the acetylcholinesterase (AchE) inhibitor donezepil. Measurements were made before and 4 h after the administration, when AchE inhibition in the cerebrospinal fluid is maximal (Rogers and Friedhoff 1998).

Statistical analysis

The electrophysiological parameters of the MB-positive group, the MB-negative group and the control group were compared by means of analysis of variance (ANOVA) F tests and t tests. Similar analyses were performed to explore the correlation between MMSE scores and the presence of MBs. Where of interest, Bonferroni corrected post hoc comparisons were conducted.

The effects of donezepil on RMT and SAI were assessed by means of a paired t test. p value <0.05 was taken as the significant threshold for all tests.

For the SvaD patients, the relation between SAI and neuropsychological tests (MMSE total score and subscores) was studied using the Pearson correlation coefficient and the Spearman rank correlation coefficient. Then, multiple linear regression was used to determine the correlation between SAI, presence of MBs and neuropsychological tests (MMSE total score and subscores), corrected for partial confounding by demographic variables and the remaining MRI findings (WMHs, PHVs, lacunar infarcts).

We considered the linear model with SAI as dependent variable (y_i), MB-positive group indicator (MB_i) and MMSE score (S_i) as explanatory variables with interaction:

$$y_i = \beta_0 + \beta_1 MB_i + \beta_2 S_i + \beta_3 MB_i S_i + \varepsilon_i \quad (1)$$

where ε_i are i.i.d. Gaussian error term. Significant interaction coefficient β_3 implies different regressions of SAI on MMSE for the two MB groups. Correction for partial confounding by demographic variables and MRI measurements reported in Table 1 was accomplished by their introduction as extra-explanatory variables in model (1) one at the time.

Results

SAI values of the SVaD patients and the control subjects are shown in the Table 2. ANOVA F test showed a significant difference between the mean amount (denoted by μ) of SAI among the three groups ($F_{(2,45)} = 46.729$, $p < 0.0001$). Since the MB-negative group displayed a larger variability than the other two groups, we used t test with Welch approximation for post hoc comparison. SAI was significantly reduced in the MB-positive group ($\mu_{pos} = 83.0\%$) than in the MB-negative group ($\mu_{neg} = 68.8\%$) and in the normal controls ($\mu_{contr} = 45.85\%$). Multiple t tests showed that $\mu_{pos} > \mu_{neg} > \mu_{contr}$, ($p = 0.008$ after Bonferroni correction).

Table 2 SAI (% of test response) of the SVaD patients and control subjects

MB-negative group		MB-positive group		Control group	
Patient	SAI	Patient	SAI	Subject	SAI
1	51	1	72	1	48
2	70	2	85	2	55
3	74	3	88	3	35
4	49	4	94	4	51
5	88	5	74	5	53
6	46	6	67	6	35
7	84	7	86	7	57
8	82	8	92	8	39
9	59	9	85	9	45
10	55	10	84	10	52
11	82	11	82	11	44
12	79	12	88	12	31
13	84	13	82	13	54
14	49			14	47
15	80			15	43
				16	40
				17	62
				18	36
				19	55
				20	35

RMT, CMCT, SICI, ICF, CMAP and F wave did not differ significantly between the two patient groups and the control group (ANOVA not significant for all F tests). All neurophysiological data are summarized in the Table 3.

RMT was not significantly modified by the administration of a single oral dose of donezepil ($p = 0.17$, paired t test). In contrast, SAI was significantly increased after donezepil administration (the mean amplitude of the conditioned response was 85.62% of the control size before the administration and 64.50% after the administration of donezepil; $p < 0.001$, paired t test).

Differences in MMSE (total score and subscores) between MB-negative group, MB-positive group and control group were all significant (Table 4); t tests showed significant differences for MMSE total score, "orientation" and "attention and calculation" subscores between the two MB groups ($p = 0.004$, 0.01 and 0.0014, respectively).

In the SVaD patients, SAI was positively correlated with MMSE total score and the 5 subscores: reduced SAI values were associated with lower scores in the neuropsychological tests (Table 5). Estimation of model (1) with S_i as MMSE total score showed that the regression coefficients are all statistically significant with $\beta_3 > 0$ ($p = 0.003$). The

Table 3 Explanatory comparison of the neurophysiological data of the SvaD patients (MB-positive and MB-negative patient groups) and the control subjects

	MB-positive group		MB-negative group		Control group		$F(2,45)$
	Mean	(SD)	Mean	(SD)	Mean	(SD)	p value
SAI (% of test response)	**83.00**	**(7.80)**	**68.80**	**(15.46)**	**45.85**	**(8.90)**	***<0.001***
RMT (% of MSO)	48.92	(8.74)	48.27	(7.80)	47.90	(8.51)	*0.9427*
CMCT (ms)	6.18	(0.48)	6.20	(0.56)	6.09	(0.61)	*0.8238*
SICI (% of test response)[a]	34.92	(9.80)	35.80	(11.49)	34.35	(10.37)	*0.8853*
ICF (% of test response)[b]	117.31	(26.44)	118.27	(27.20)	116.65	(26.22)	*0.9601*
CMAP (mV)	7.67	(0.73)	7.61	(0.96)	7.63	(0.95)	*0.9826*
F wave (μV)	254.92	(50.89)	240.80	(54.40)	242.35	(45.80)	*0.7159*

Bold type indicates significant differences among the three groups

SAI short latency afferent inhibition, *RMT* resting motor threshold, *MSO* maximum stimulator output, *CMCT* central motor conduction time, *SICI* short latency intracortical inhibition, *ICF* intracortical facilitation, *CMAP* compound muscle action potential

[a] Grand mean of the SICI at the three ISIs studied

[b] Grand mean of the ICF at the three ISIs studied

Table 4 Mini-Mental State Examination (MMSE) total score and subscores in the MB-positive and MB-negative groups

	MB-negative group		MB-positive group		Control group		$F(2,45)$
	Mean	(SD)	Mean	(SD)	Mean	(SD)	p value
MMSE total	22.07	(1.67)	19.92	(1.93)	29.65	(0.59)	***<0.001***
MMSE orientation	8.33	(0.72)	7.62	(0.65)	10.00	(0.00)	***<0.001***
MMSE immediate	2.60	(0.51)	2.38	(0.65)	3.00	(0.00)	***<0.001***
MMSE atten. and calc.	1.60	(0.63)	0.77	(0.60)	4.85	(0.37)	***<0.001***
MMSE delayed rec.	1.87	(0.35)	1.62	(0.51)	2.85	(0.37)	***<0.001***
MMSE language	7.67	(0.82)	7.54	(0.66)	8.95	(0.22)	***<0.001***

Bold type indicates significant differences between the two patient groups

Table 5 Correlation between SAI and MMSE total score and subscores in the patients with SVaD evaluated by means of Pearson and Spearman correlation coefficients

	MMSE total	MMSE orientation	MMSE immediate rec.	MMSE atten. and calc.	MMSE delayed rec.	MMSE language
Pearson						
Estimate	**−0.861**	**−0.794**	**−0.417**	**−0.523**	−0.314	**−0.552**
p value	***<0.0001***	***<0.0001***	***0.027***	***0.004***	*0.104*	***0.002***
Spearman						
Estimate	**-0.896**	**−0.774**	**−0.397**	**−0.553**	−0.353	**−0.620**
p value	***<0.0001***	***<0.0001***	***0.036***	***0.002***	*0.065*	***<0.001***

Bold type indicates significant correlation

hypothesis of normality of the residuals is respected as shown by the Kolmogorov–Smirnov test (H_0: $\varepsilon_i \sim N(0,1)$, $p = 0.56$). We plot the data in two panels that show separate fitted regression lines in Fig. 1. Notably, the correlation between SAI and MMSE was negative in both patient groups (as shown by the slopes $\beta_2 < 0$ and $\beta_2 + \beta_3 < 0$) and the regression line of MMSE to SAI was steeper for the MB-negative group.

Finally, adjustment for age, gender, education, presence of lacunae, severe PVH and severe WMH did not affect the estimation results of model (1): when added as explanatory variables, estimation showed no significant effect, and the

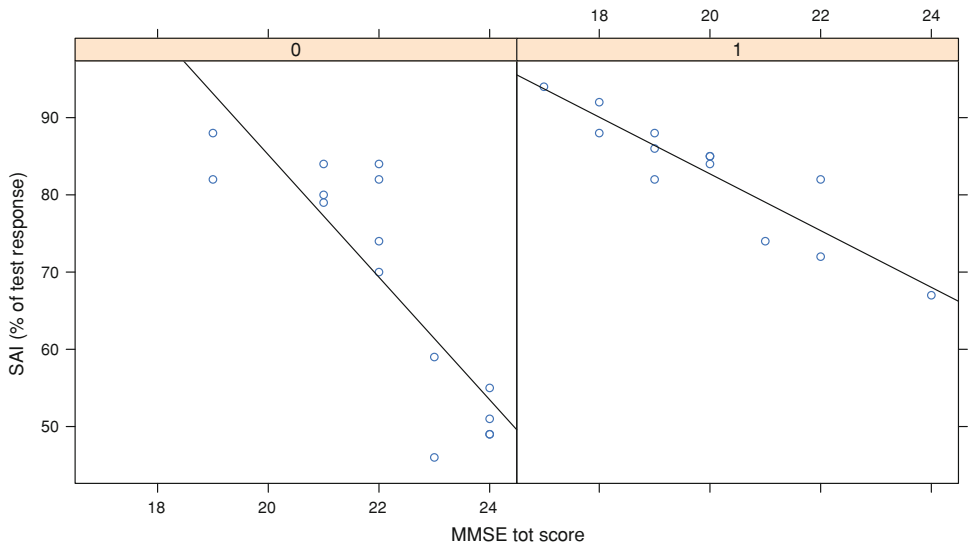

Fig. 1 Data on patients with SVaD showing the correlation between MMSE total score and SAI by estimation of the multiple linear regression model (1). *Left panel* MB-negative group, *right panel* MB-positive group

significant relationship between MMSE, MB grouping and SAI was not influenced by their introduction.

Discussion

We first explored the relationship between MBs, SAI and cognitive function in patients with SvaD. Our study shows that SAI, a putative marker of cholinergic cortical activity, is significantly more abnormal in the patients with SVaD and MBs than in SVaD patients without MBs who also present a significantly reduced SAI as compared with normal controls.

The recently reported interaction of SAI with the other inhibitory process N100 EEG response has provided further evidence of the cortical origin of SAI (Bikmullina et al. 2009). Moreover, we failed to find in the present study significant differences in F wave between groups that might be expected if spinal mechanisms are contributory.

SAI is thought to be related to central cholinergic activity because in normal subjects it can be reduced or abolished by intravenous injection of the muscarinic antagonist scopolamine (Di Lazzaro et al. 2000) and is modulated by Ach in healthy subjects (Di Lazzaro et al. 2005a, 2006; Fujiki et al. 2006). SAI is also influenced by GABAergic drugs such as some benzodiazepines in healthy subjects (Di Lazzaro et al. 2005a, b, c) and by dopaminergic drugs in patients with Parkinson disease (Sailer et al. 2003). Preliminary data suggest that other neurotransmitters/neuromodulators are likely not involved in the regulation of SAI in that quetiapine, an antagonist at multiple neurotransmitter receptors in the brain such as serotonin 5HT1A and 5HT2, dopamine D1 and D2, histamine, and adrenergic $\alpha 1$ and $\alpha 2$ receptors, does not modify SAI in healthy subjects (Di Lazzaro et al. 2005b). Therefore, SAI is thought to represent a non-invasive way of testing the integrity of some cholinergic cortical circuits (Ziemann 2004), while the contribution of neurotransmitters other than Ach is not well understood. On the other hand, it has been recently reported that dopamine modifies SAI in AD (Martorana et al. 2009). Therefore, it should be considered that other neurotransmitters such as dopamine may be able to modulate cortical cholinergic function in AD patients.

Interestingly, administration of a single dose of the AchE inhibitor donezepil improved SAI in our patients, similarly to that previously reported in AD patients. These results suggest that the evaluation of the effects of the AchE inhibitors on SAI could be useful in the management of SvaD patients (similar to that reported in patients with AD) because it is currently impossible to predict an individual therapeutic response in these patients.

We cannot rule out the possibility that in some patients the cholinergic dysfunction was due to a concurrent AD pathology. The patients with abnormal SAI could have concomitant neuropathological changes of AD and thus represent the percentage of patients with a mixed form of dementia. Indeed, neuropathological studies of VD have demonstrated that 25–30% of patients with the clinical diagnosis of VD show the concomitant neuropathology of AD (Kalaria and Ballard 1999; Vinters et al. 2000). However, SvaD and AD can be distinguished by the mode of onset and progression of the cortical deficits. Memory impairment, usually the first and more severe cognitive manifestation of AD, was quite mild in our patients while they display significant poor performance on "attention and calculation" tasks, similar to that previously reported (Yakushiji et al. 2008).

A possible explanation for the SAI abnormality is that the subcortical lesions in SVaD interrupt ascending cholinergic axons determining cortical cholinergic

denervation. White matter lesions may directly affect cholinergic projection (Selden et al. 1998; Swartz et al. 2003), and preclinical (Togashi et al. 1994; Kimura et al. 2000) as well clinical evidence (Gottfries et al. 1994; Martin-Ruiz et al. 2000) suggest that the cholinergic system might also be involved in SVaD. Mesulam et al. (2003) demonstrated in a patient with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) that pure white matter infarcts, similar to those seen in SVaD, can cause cortical cholinergic denervation, but in patterns that vary from those seen in AD. Interestingly, in CADASIL patients the amount of SAI was found to be significantly smaller than in normal subjects (Manganelli et al. 2008).

Clinical implications of MBs have rarely been studied. Despite considerable interest in MBs as a marker of bleeding-prone small-vessel angiopathy, they have initially been considered to be clinically silent (Kwa et al. 1998; Kato et al. 2002; Tsushima et al. 2003). However, since they are located in widespread cortical and basal ganglia regions and are histologically characterized by tissue damage, they could cause cognitive dysfunction. Moreover, histopathological data that MBs show not only haemosiderin deposition but also surrounding gliosis, and sometimes frank necrosis or infarction also support the clinical importance of MBs (Tanaka et al. 1999).

Werring et al. (2004) found that stroke patients with MBs were more impaired at frontal executive functions than those without MBs, and postulated that MBs located in the frontal lobe and the basal ganglia might have caused it. There were two studies that investigated MBs in patients with vascular dementia (Hanyu et al. 2003; Cordonnier et al. 2006). One study enrolled 31 patients with vascular dementia (not necessarily SvaD) and reported the frequency of MBs of 65% (Cordonnier et al. 2006); the other study that involved Asian SvaD patients reported the frequency of 77% (Hanyu et al. 2003). Moreover, a recent review article suggested that the cognitive impairment of patients with cerebral amyloid angiopathy (CAA) might be associated with the number of baseline hemorrhages (Viswanathan and Chabriat 2006). The number of microhemorrhages in the frontal lobes and basal ganglia was the only independent predictor of executive dysfunction in the CAA patients; however, the investigators did not evaluate all potential confounders, particularly the number or location of associated lacunar infarctions. In agreement with our findings, another study showed that MBs affect the general cognitive dysfunction and the severity of dementia in patients with SvaD (Won Seo et al. 2007). Yakushiji et al. (2008) recently also reported that MBs appear to be primarily associated with global cognitive dysfunction.

The results of the present study are thus consistent with the more recent literature on the clinical significance of the MBs in SVaD and further support the notion that not only ischemia but also MBs are primary pathomechanisms of cognitive impairment. Only in CADASIL patients MBs were previously found not to be associated with cognitive dysfunction (Liem et al. 2007). This finding could be explained by the more extensive ischemic lacunar changes occurring at a younger age in CADASIL.

The mechanisms underlying the pathological association between MBs and cognitive dysfunction remain still unclear. A histopathologic study on MBs in patients with primary cerebral hemorrhage has shown that the presence of MBs indicated widespread damage of arterioles by hypertension or amyloid deposition (Fazekas et al. 1999). The location of brain MBs may be of importance, with cortico-subcortical brain MBs being more strongly related to CAA than brain MBs in deep of infratentorial locations (Cordonnier and van der Flier 2011); however, this issue has not been addressed in this preliminary study. Anyway, the presence of MBs may thus imply much more severe disruption of the neural network between cortical and subcortical structures than ischemic SVDs. MBs would be expected to cause cognitive impairment if they disrupt strategically important white matter tracts or eloquent cortical areas.

Since MBs are particularly common in the white matter regions (Offenbacher et al. 1996), it could be hypothesized that executive functions would be most affected, due to disruption of frontal–basal ganglia connections.

Interestingly, patients with Parkinson's disease and dementia have also been shown to display significantly poor performance on "attention and calculation" tasks in the MMSE (Yakushiji et al. 2008). This cognitive deficit is considered to result from severe dysfunction of cholinergic pathways in the frontal-subcortical circuits (Bohnen et al. 2003, 2006). In common with previous studies, we observed MBs most frequently in the basal ganglia as well as in the frontal regions. Predominant occurrence of MBs in these regions may thus cause executive dysfunction (Werring et al. 2004). This hypothesis requires confirmation by future studies employing more detailed neuropsychological tests which offer higher sensitivity for the assessment of cognitive functions (in particular the executive functions) in patients with SvaD (O'Sullivan et al. 2005). The association we have found between MMSE scores and the presence of MBs as well as with SAI values would be more even prominent if it would have used more sophisticated neuropsychological testing.

Our data indicate that the cumulative effect of MBs on cognition appears to be independent of coexisting ischaemic cerebrovascular disease, and in particular is independent of the severity of ischaemic SVaD as assessed by MRI white matter changes. The finding that MBs are associated with cholinergic dysfunction has potential diagnostic and

therapeutic implications. It would be of particular interest to explore in future studies the responsiveness of patients with MBs, stratified according to their cholinergic dysfunction, defined using SAI, to AchE inhibitors.

TMS studies may be important for evaluating the causes of cognitive impairment in cerebrovascular disease. Thus, in patients with cerebrovascular risk factors and cognitive impairment, T2*-weighted GE-MRI may be a helpful adjunct to standard MRI in clarifying the mechanism of cognitive impairment.

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