



Genotype (cystatin C) and EEG phenotype in Alzheimer disease and mild cognitive impairment: A multicentric study

Claudio Babiloni^{a,b,c,*}, Luisa Benussi^b, Giuliano Binetti^b, Paolo Bosco^d,
Gabriella Busonero^b, Simona Cesaretti^c, Gloria Dal Forno^e, Claudio Del Percio^{a,b,c},
Raffaele Ferri^d, Giovanni Frisoni^{b,c}, Ghidoni Roberta^b,
Guido Rodriguez^f, Rosanna Squitti^c, and Paolo M. Rossini^{b,c,d}

^aDip. Fisiologia Umana e Farmacologia, Univ. "La Sapienza" Rome, Italy

^bA.Fa.R.-IRCCS "S. Giovanni di Dio-F.B.F.", Brescia, Italy

^cA.Fa.R., Dip. Neurosci. Osp. FBF; Isola Tiberina, Rome, Italy

^dDept. of Neurology, Oasi Institute for Research on Mental Retardation and Brain Aging (IRCCS), Troina, Italy

^eClin. Neurol. University "Campus Biomedico" Rome, Italy

^fDivision of Clinical Neurophysiology (DISEM), University of Genova, Italy

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Previous findings demonstrated that haplotype B of CST3, the gene coding for cystatin C, is a recessive risk factor for late-onset Alzheimer's disease (AD; Finckh, U., von der Kammer, H., Velden, J., Michel, T., Andresen, B., Deng, A., Zhang, J., Muller-Thomsen, T., Zuchowski, K., Menzer, G., Mann, U., Papassotiropoulos, A., Heun, R., Zurdel, J., Holst, F., Benussi, L., Stoppe, G., Reiss, J., Miserez, A.R., Staehelin, H.B., Rebeck, G.W., Hyman, B.T., Binetti, G., Hock, C., Growdon, J.H., Nitsch, R.M., 2000. Genetic association of the cystatin C gene with late-onset Alzheimer disease. *Arch. Neurol.* 57, 1579–1583). In the present multicentric electroencephalographic (EEG) study, we analyzed the effects of CST3 haplotypes on resting cortical rhythmicity in subjects with AD and mild cognitive impairment (MCI) with the hypothesis that sources of resting EEG rhythms are more impaired in carriers of the CST3 B haplotype than non-carriers. We enrolled a population of 84 MCI subjects (42% with the B haplotype) and 65 AD patients (40% with the B haplotype). Resting eyes-closed EEG data were recorded in all subjects. EEG rhythms of interest were delta (2–4 Hz), theta (4–8 Hz), alpha 1 (8–10.5 Hz), alpha 2 (10.5–13 Hz), beta 1 (13–20 Hz), and beta 2 (20–30 Hz). EEG cortical sources were estimated by low-resolution brain electromagnetic tomography (LORETA). Results showed that the amplitude of alpha 1 (parietal, occipital, temporal areas) and alpha 2 (occipital area) was statistically lower in CST3 B carriers than non-carriers ($P < 0.01$). Whereas there was a trend towards statistical significance that amplitude of occipital delta sources was stronger in CST3 B carriers than in non-carriers. This was true for both MCI and AD subjects. The present findings represent the first demonstration of

relationships between the AD genetic risk factor CST3 B and global neurophysiological phenotype (i.e., cortical delta and alpha rhythmicity) in MCI and AD subjects, prompting future genotype–EEG phenotype studies for the early prediction of AD conversion in individual MCI subjects.

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Introduction

Mild cognitive impairment (MCI) is a definition encompassing the clinical state between elderly normal cognition and dementia, when somebody has memory complaints and objective evidence of cognitive impairment, but no evidence of dementia (Flicker et al., 1991; Petersen et al., 1995, 2001). This condition is considered as a precursor of Alzheimer's disease – AD – (Galluzzi et al., 2001; Scheltens et al., 2002; Arnaiz and Almkvist, 2003), since recent studies have shown a high rate of progression to AD in subjects affected by MCI (Bachman et al., 1993; Gao et al., 1998; Petersen et al., 2001). In normal aging (with no MCI symptoms), annual conversion rate to AD ranges from 0.17% at age 65–69 to 3.86% at age 85–89, whereas the cumulative rate for all dementias ranges from 0.82% to 5.33% (Petersen et al., 2001; Frisoni et al., 2004). The annual conversion rate from MCI to AD is instead much higher, ranging from 6 to 25% (Petersen et al., 2001). Furthermore, a recent longitudinal study has shown that MCI converts to AD at a rate of 12% per year (Petersen et al., 1999), and, by the end of 6 years,

* Corresponding author. Dipartimento di Fisiologia Umana e Farmacologia, Università degli Studi di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Rome, Italy. Fax: +39 06 49910917.

E-mail address: claudio.babiloni@uniroma1.it (C. Babiloni).

URL: <http://hreeg.ifu.uniroma1.it> (C. Babiloni).

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approximately 80% of MCI subjects have developed AD. Taken together, these data suggest the hypothesis that, in the majority of cases, MCI is a transition state on a linear progression towards AD. “Transition” hypothesis, however, is challenged by observations indicating that not all MCI patients deteriorate over time (Bennett et al., 2002; Larrieu et al., 2002). In fact, the risk of AD in MCI subjects has been shown to be higher than in normal controls only during the first 4 years of follow up (Bennett et al., 2002). Furthermore, previous evidence has indicated that cumulative conversion rates from MCI to AD have ranged from 40% to 60%, depending on the studies (Bennett et al., 2002; Larrieu et al., 2002; Fisk et al., 2003). Practical consequence of these observations is that a diagnosis of MCI might be associated with indexes able to indicate the likelihood of the conversion to dementia. In all cases, early identification of MCI patients is an important preliminary aim of research in this field (Braak and Braak, 1991; Rogers et al., 1996; Small et al., 1997).

Previous studies have investigated the electrophysiological substrate of AD. In mild AD, electroencephalographic (EEG) rhythms differ from normal elderly (Nold) and vascular dementia subjects, AD patients being characterized by excessive delta (0–3 Hz) and a significant decrement of posterior alpha rhythms (8–12 Hz; Dierks et al., 1993, 2000; Huang et al., 2000; Ponomareva et al., 2003; Babiloni et al., 2004a). EEG rhythms abnormality in dementia has been associated with altered regional cerebral blood flow (rCBF)/metabolism and cognitive function as screened by the Mini Mental State Examination—MMSE (Passero et al., 1995; Sloan et al., 1995; Rodriguez et al., 1998, 1999a,b; Nobili et al., 2002a,b). Similarly, MCI subjects have shown increase of theta (4–7 Hz) as well as decrease of alpha power (Jelic et al., 1996; Huang et al., 2000; Grunwald et al., 2001; Frodl et al., 2002), when compared to normal elderly (Nold). These EEG parameters have presented an intermediate magnitude in MCI subjects with respect to those observed in Nold and dementia patients (Elmstahl and Rosen, 1997; Huang et al., 2000; Jelic et al., 2000).

Despite the converging evidence of abnormal cortical rhythms in MCI and AD, EEG findings alone seem to be insufficient to predict conversion of MCI to dementia. It is reasonable that additional biological parameters are needed to this purpose. In this respect, a strict relationship between apolipoprotein E (ApoE) genotype and late-onset AD is now widely accepted (Bunce et al., 2004; Saunders, 2001; Small, 1996; Lee et al., 2003; Crutcher, 2004; Huang et al., 2004; Lahiri et al., 2004; Mosconi et al., 2004; Qiu et al., 2004). Of note, ApoE ϵ 4 affects EEG rhythms in AD (Jelic et al., 1997; Lehtovirta et al., 1995, 1996, 2000). Compared to AD patients with ϵ 2 or ϵ 3, AD patients with ϵ 4 had higher delta (1.5–3.9 Hz) and theta (4.1–7.3 Hz) EEG power as well as lower alpha (7.6–13.9 Hz) and beta (14.2–20 Hz) EEG power (Lehtovirta et al., 1995, 1996, 2000). In addition, recent evidence from our group (Babiloni et al., *in press-a*) showed that, in MCI and AD groups, the amplitude of alpha sources (8–13 Hz) in occipital, temporal, and limbic areas was lower in ϵ 4 allele carriers than in non-carriers.

In addition to ApoE, the gene coding for cystatin C (CST3) has also been associated with increased risk of AD (Crawford et al., 2000; Finckh et al., 2000; Beyer et al., 2001; Olson et al., 2002; Lin et al., 2003; Goddard et al., 2004), even if this finding was not confirmed in different populations (Roks et al., 2001; Maruyama et al., 2001; Dodel et al., 2002). Cystatin C is an endogenous cysteine proteinase inhibitor belonging to the type 2 cystatin superfamily. The mature, active form of human cystatin C is a single non-glycosylated polypeptide chain consisting of 120-amino-acid

residues, with a molecular mass of 13,343–13,359 Da. It contains four characteristic disulfide-paired cysteine residues. Human cystatin C is encoded by the CST3 gene, ubiquitously expressed at moderate levels. The cystatin C monomer is present in all human body fluids, and it is preferentially abundant in cerebrospinal fluid, seminal plasma, and milk (Mussap and Plebani, 2004).

CST3 is present within the population in two common haplotypes (named A and B in Finckh et al., 2000) differing for three strongly genetically linked base substitution in the 3' region of the gene, one situated in the coding region at position 1 of codon 25 (Ala25). The haplotype B, containing nucleotide A at this position (Thr25), has been proposed as a recessive risk factor for late-onset AD in patients older than 75 years, independently of ApoE genotype (Finckh et al., 2000; Lin et al., 2003). Functional characterization of this genetic variant showed that CST3 haplotype B is associated with a reduction in the level of cystatin C secretion (Benussi et al., 2003).

Immunohistochemical studies in AD brains have shown that cystatin C protein co-localizes with beta amyloid (Vinters et al., 1990; Levy et al., 2001; Sastre et al., 2004); it accumulates within intracellular vesicles in susceptible neurons (Deng et al., 2001); and it has a proliferative effect on neural stem cells in vitro and in vivo (Taupin et al., 2000). A decrease of cystatin C levels in the brain might therefore result both in lack of protection from toxic insults and in a defective stem cells-mediated regeneration.

To our knowledge, the potential relationship between CST3 levels and the characteristics of EEG rhythmicity have not been previously explored in either MCI or in AD subjects. In this investigation, we tested whether the presence of risk factor B CST3 haplotype might affect EEG rhythmicity sources in MCI and AD. Cortical sources of the EEG rhythms were estimated by low-resolution brain electromagnetic tomography (LORETA; Pascual-Marqui and Michel, 1994; Pascual-Marqui et al., 1999, 2002), which has been successfully used in recent studies on physiological and pathological aging (Dierks et al., 2000; Babiloni et al., 2004a, *in press-a*).

Methods

Part of the procedures (EEG recordings and LORETA analysis) pertinent to the current study as well as a description of the potential meaning of cortical rhythms in aging have been extensively described in 3 recent papers (Babiloni et al., 2004a, *in press-a*). However, it should be stressed that the aims of the previous and current studies are different. The previous studies aimed at analyzing (i) the distributed cortical EEG sources specific to mild AD subjects compared to vascular dementia (VaD) and normal elderly subjects (Babiloni et al., 2004a), (ii) the effects of the ApoE ϵ 4 on distributed cortical EEG sources in MCI and AD subjects (Babiloni et al., *in press-a*), and (iii) the distributed cortical EEG sources in normal young and elderly subjects (Babiloni et al., *in press-b*). In contrast, the current study focused on the effects of the CST3 B haplotype on distributed cortical EEG sources in MCI and AD subjects.

Subjects

Eighty-four MCI subjects and 65 AD patients were enrolled. We also recruited 74 cognitively normal elderly (Nold) subjects as controls. Local institutional ethics committees approved the study.

All experiments were performed with the informed and overt consent of each participant or caregiver, in line with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and the standards established by the Author's Institutional Review Board. The AD group was sub-divided in two genetic sub-groups: AD carriers of the CST3 B haplotype (including 26 subjects with the AB genotype) and AD non-carriers of the CST3 B haplotype (including 39 subjects with the AA genotype); the MCI group was sub-divided in two genetic sub-groups: MCI carriers of the CST3 B haplotype (including 35 subjects with the AB genotype) and MCI non-carriers of the CST3 B haplotype (including 49 subjects with the AA genotype). Of note, the CST3 BB genotype is quite rare making it difficult to recruit a sufficient number of carriers of this variant. Therefore, no MCI or AD carrier of CST3 BB genotype was included in the present study.

Subjects recruitment aimed at balancing the ApoE ϵ 4 factor in CST3 AA and CST3 AB carriers. The percentage of ApoE ϵ 4 carriers was 14% in both MCI CST3 AB and CST3 AA carriers, and it was 46% in CST3 AB and CST3 AA AD carriers.

A control group of healthy elderly subjects (Nold) was also recruited (mostly among patients' spouses) in order to preliminarily ascertain that selected AD and MCI subjects presented indeed the typical EEG rhythmicity changes associated with disease and cognitive impairment. Of note, we were unable to recruit a sufficient number of Nold subjects for the extraction of Genomic DNA, since many of them refused the blood sample extraction. This made not viable the analysis of the relationship between CST3 levels and the characteristics of EEG rhythmicity in Nold subjects compared to MCI and AD subjects, an important issue for future research.

The characteristics of the recruited MCI and AD subjects are shown in Table 1. Table 1 summarizes the relevant demographic and clinical parameters of participants sub-divided in genetic sub-groups, namely, MCI/AD carriers of the CST3 B haplotype and MCI/AD non-carriers. Four ANOVA analyses using the factors Genotype (presence or absence of CST3 B) were computed to evaluate the presence or absence of statistically significant differences between MCI/AD CST3 B carriers and MCI/AD CST3 B non-carriers for age, education, gender, and MMSE. No statistically significant difference for age ($P > 0.75$), education ($P > 0.27$), gender ($P > 0.95$), and MMSE ($P > 0.55$) was found. However, age and education were used as covariates in the statistical evaluation of cortical sources of EEG rhythms to remove possible confounding effects.

Diagnostic criteria

Probable AD was diagnosed according to NINCDS-ADRDA (McKhann et al., 1984) and DSM IV criteria. All recruited AD

patients underwent general medical, neurological, and psychiatric assessments. Patients were also rated with a number of standardized diagnostic and severity instruments that included the Mini Mental State Examination (MMSE, Folstein et al., 1975), the Clinical Dementia Rating Scale (CDR, Hughes et al., 1982), the Geriatric Depression Scale (GDS, Yesavage et al., 1982–83), the Hachinski Ischemic Scale (HIS, Rosen et al., 1980), and the Instrumental Activities of Daily Living scale (IADL, Lawton and Brodie, 1969). Neuroimaging diagnostic procedures (CT or MRI) and complete laboratory analyses were carried out to exclude other causes of progressive or reversible dementias in order to have a homogenous AD patient sample. Exclusion criteria included, in particular, evidence of (i) frontotemporal dementia, (ii) vascular dementia (i.e., the vascular dementia was also diagnosed according to NINDS-AIREN criteria; Roman et al., 1993), (iii) extrapyramidal syndromes, (iv) reversible dementias, and (v) fluctuations in cognitive performance and visual hallucinations (suggestive of a possible Lewy body dementia). The detection of vascular component in dementia and MCI was accounted based on previous theoretical guidelines from our network (Frisoni et al., 1995; Galluzzi et al., 2005). The recruitment of the AD subjects was made pairing in CST3 B carriers and non-carriers the percentage of subjects underwent to therapy with acetylcholinesterase inhibitors (donepezil; 5–10 mg/day) and the total duration of that therapy. Of note, antidepressant and/or antihypertensive were suspended for about 24 h before EEG recordings, and subjects receiving such a therapy were approximately paired between CST3 B carriers and non-carriers. This was done to pair the accumulation/minimal rebound effects of the drugs between CST3 B carriers and non-carriers. Washed out of the drugs would have required a too long suspension with high risks for the patients.

Inclusion and exclusion criteria for MCI diagnosis aimed at selecting elderly persons with objective cognitive deficits, especially in the memory domain, who did not meet criteria for dementia or AD (Albert et al., 1991; Flicker et al., 1991; Zaudig, 1992; Devanand et al., 1997; Petersen et al., 1997, 2001). Inclusion criteria for MCI subjects were (i) objective memory impairment on neuropsychological evaluation, as defined by performances ≥ 1.5 standard deviation below age and education-matched controls; (ii) normal activities of daily living as documented by the history and evidence of independent living; and (iii) a clinical dementia rating score of 0.5. Exclusion criteria for MCI were (i) AD, as diagnosed by the procedures described above; (ii) evidence of other concomitant dementias such as frontotemporal, vascular dementia, reversible dementias, fluctuations in cognitive performance, and/or features of mixed dementias; (iii) evidence of concomitant extrapyramidal symptoms; (iv) clinical and indirect evidence of depression as revealed by GDS scores greater than 14; (v) other

Table 1
Demographic and neuropsychological data of participants

	MCI		AD	
	CST3 B non-carriers	CST3 B carriers	CST3 B non-carriers	CST3 B carriers
<i>N</i>	49	35	39	26
Age (years)	70.6 (± 1.1 SE) 54 to 87	69.7 (± 1.4 SE) 53 to 81	75.3 (± 1 SE) 64 to 87	74.8 (± 1.7 SE) 54 to 86
Gender (M/F)	19/30 (39%)	14/21 (40%)	10/29 (26%)	7/19 (28%)
MMSE	25.9 (± 0.4 SE) 20.9 to 30	25.7 (± 0.4 SE) 20.2 to 29	19.6 (± 0.5 SE) 15.3 to 29	19.6 (± 0.8 SE) 13 to 28.3
Education (years)	7.4 (± 0.6 SE) 2 to 18	7.3 (± 0.8 SE) 3 to 18	6.3 (± 0.5 SE) 3 to 13	6.6 (± 0.8 SE) 3 to 13
ApoE ϵ 4 (%)	14%	14%	46%	46%

Nold—normal elderly; MCI—mild cognitive impairment; AD—Alzheimer's disease.

psychiatric diseases, epilepsy, drug addiction, alcohol dependence, and use of psychoactive drugs or drugs interfering with brain cognitive functions including acetylcholinesterase inhibitors; and (vi) current or previous systemic diseases (including diabetes mellitus) or traumatic brain injuries.

All Nold subjects underwent physical and neurological examinations as well as cognitive screening (including MMSE and GDS). Subjects affected by chronic systemic illnesses (i.e., diabetes mellitus or organ failure) were excluded, as were subjects receiving psychoactive drugs. Subjects with a history of present or previous neurological or psychiatric disease were also excluded. All Nold subjects had a GDS score lower than 14.

ApoE and CST3 genotyping

Genomic DNA was extracted from whole-blood samples of AD patients and MCI subjects according to standard procedures. APOE genotyping was carried out by PCR amplification and HhaI restriction enzyme digestion. The genotype was resolved on 4% Metaphor Gel (BioSpa, Italy) and visualized by ethidium bromide staining (Hixson and Vernier, 1990). DNA from subjects were analyzed for CST3 haplotypes at the 5' end, as previously described (Finckh et al., 2000).

EEG recordings

EEG data were recorded in resting subjects (eyes closed) by specialized clinical units. EEG recordings were performed (0.3–70 Hz bandpass) from 19 electrodes positioned according to the International 10–20 System (i.e., Fp1, Fp2, F7, F3, Fz, F4, F8, T3, C3, Cz, C4, T4, T5, P3, Pz, P4, T6, O1, O2). A specific reference electrode was not imposed to all recording units of this multicentric study, since preliminary data analysis and LORETA source analysis were carried out after EEG data were re-referenced to a common average reference. To monitor eye movements, the horizontal and vertical electrooculogram (0.3–70 Hz bandpass) was also collected. All data were digitized in continuous recording mode (5 min of EEG; 128- to 256-Hz sampling rate). In all subjects, EEG recordings were performed in the late morning. State of vigilance was controlled by on-line visual inspection of EEG traces, and subjects' drowsiness was avoided by verbal warnings. Of note, EEG recordings lasting 5 min allowed the comparison of the present results with several previous AD studies using either EEG recording periods shorter than 5 min (Pucci et al., 1999; Szelies et al., 1999; Rodriguez et al., 2002; Babiloni et al., 2004a,b) or shorter than 1 min (Dierks et al., 1993, 2000). Longer resting EEG recordings in AD patients would have reduced data variability but would have increased the possibility of EEG rhythmic oscillations slowing because of reduced vigilance and arousal.

The EEG data were analyzed and fragmented off-line in consecutive epochs of 2 s. For standardization purposes, preliminary analysis of all data was centralized in one research unit. The EEG epochs with ocular, muscular, and other types of artifact were preliminary identified by a computerized automatic procedure. EEG epochs with sporadic blinking artifacts (less than 15% of the total) were corrected by an autoregressive method (Moretti et al., 2003). Two independent experimenters manually confirmed the EEG segments accepted for further analysis. Of note, they were blind to the diagnosis at the time of the EEG data analysis. Indeed, the diagnosis required the integration of many other clinical and

psychometric parameters and was formulated several weeks after the EEG data recording and analysis. The mean of the individual artifact-free EEG epochs was 134 (± 10 standard error, SE) in Nold group, 132 (± 5 SE) in MCI group, and 131 (± 5 SE) in AD group. In the two MCI sub-groups, the mean of the individual artifact-free EEG epochs was 130 (± 7 SE) in MCI CST3 B non-carriers and 134 (± 7 SE) in MCI CST3 B carriers. In the two AD sub-groups, the mean of the individual artifact-free EEG epochs was 128 (± 6 SE) in AD CST3 B non-carriers and 135 (± 17 SE) in CST3 B carriers.

Spectral analysis of the EEG data

The spectral analysis of the EEG data was computed with the original LORETA software (<http://www.unizh.ch/keyinst/NewLORETA/LORETA01.htm>). A digital FFT-based power spectrum analysis (Welch technique, Hanning windowing function, no phase shift) computed power density of the EEG rhythms with 0.5-Hz frequency resolution from the common averaged EEG potentials. The output of the procedure was the EEG cross-spectra. The following standard band frequencies were studied: delta (2–4 Hz), theta (4–8 Hz), alpha 1 (8–10.5 Hz), alpha 2 (10.5–13 Hz), beta 1 (13–20 Hz), and beta 2 (20–30 Hz). These band frequencies were chosen averaging those used in previous relevant EEG studies on dementia (Jelic et al., 1996; Chiaramonti et al., 1997; Rodriguez et al., 1999a,b) and have been successfully used in recent studies on AD of this Consortium (Babiloni et al., 2004a,b). Sharing of a frequency bin by two contiguous bands is a widely accepted procedure (Cook and Leuchter, 1996; Jelic et al., 1996; Nobili et al., 1998; Pucci et al., 1997; Kolev et al., 2002; Holschneider et al., 1999). Furthermore, this fits the theoretical consideration that near EEG rhythms may overlap at their frequency borders (Klimesch, 1996, 1999; Klimesch et al., 1997, 1998; Babiloni et al., 2004c,d,e,f,g, 2005).

Choice of fixed EEG bands did not account for individual alpha frequency (IAF) peak, defined as the frequency associated with the strongest EEG power at the extended alpha range (Klimesch, 1999). However, this should not affect the results, since most of the subjects had IAF peaks within the alpha 1 band (8–10.5 Hz). In particular, mean IAF peak was 9.3 Hz (± 0.1 standard error, SE) in Nold subjects, 9.1 Hz (± 0.1 SE) in MCI subjects, and 8.2 Hz (± 0.2 SE) in AD patients. In the two MCI sub-groups, the mean IAF peak was 9.2 Hz (± 0.2 SE) in MCI CST3 B haplotype non-carriers and 9 Hz (± 0.2 SE) in MCI CST3 B carriers. In the two AD sub-groups, the mean IAF peak was 8.3 Hz (± 0.2 SE) in AD CST3 B haplotype non-carriers and 8 Hz (± 0.2 SE) in CST3 B carriers. To control for the effect of IAF on the EEG comparisons between these two sub-groups, the IAF peak was used as a covariate (together with age and education) for further statistics.

We could not use narrow frequency bands for beta 1 (13–20 Hz) and beta 2 (20–30 Hz) because of the variability of beta peaks in the power spectra. Therefore, LORETA results for the beta bands could suffer from sensitivity limitations of EEG spectral analyses for large bands (Szava et al., 1994).

Cortical source analysis of EEG rhythms by LORETA

The EEG cross-spectra were given as an input to the proper option of the original LORETA software for the EEG source analysis (Pascual-Marqui and Michel, 1994; Pascual-Marqui et al., 1999, 2002). LORETA is a functional imaging technique belonging to a family of linear inverse solution procedures (Valdes et al., 1998),

which model 3D distributions of the cortical source patterns generating scalp EEG data (Pascual-Marqui et al., 2002). With respect to the dipole modeling of cortical sources, no a priori decision of the dipole position is required by the investigators in LORETA estimation. In a previous review paper, it has been shown that LORETA was quite efficient when compared to other linear inverse algorithms like minimum norm solution, weighted minimum norm solution, and weighted resolution optimization (Pascual-Marqui et al., 1999). Furthermore, independent validation of LORETA solutions has been provided by recent studies (Phillips et al., 2002; Yao and He, 2001). Finally, LORETA has been successfully used in recent EEG studies on pathological aging (Dierks et al., 2000; Babiloni et al., 2004a).

LORETA computes 3D linear solutions (LORETA solutions) for the EEG inverse problem within a three-shell spherical head model including scalp, skull, and brain compartments. The brain compartment is restricted to the cortical gray matter/hippocampus of a head model co-registered to the Talairach probability brain atlas and digitized at the Brain Imaging Center of the Montreal Neurological Institute (Talairach and Tournoux, 1988). This compartment includes 2394 voxels (7-mm resolution), each voxel containing an equivalent current dipole.

LORETA can be used from data collected by low spatial sampling of 10–20 System (19 electrodes) when cortical sources are estimated from resting EEG rhythms. Several previous studies have shown that these rhythms are generated by largely distributed cortical sources that can be accurately investigated by standard 10–20 System and LORETA (Anderer et al., 2003, 2004; Isotani et al., 2001; Laufer and Pratt, 2003; Babiloni et al., 2004a; Veiga et al., 2003).

The LORETA solutions consisted of voxel spectral density of estimated z -current density values able to predict EEG spectral data at scalp electrodes. These solutions are reference free in that one obtains the same LORETA solutions for EEG data referred to any reference electrode including common average. A normalization of the data was obtained by dividing the LORETA solutions at each voxel with the LORETA solution averaged across all frequencies (0.5–45 Hz) and across all 2394 voxels of the brain volume. After the normalization, the LORETA solutions lost the original physical dimension and were represented by an arbitrary unit scale. In the “Methods” and “Results” sections, we used “LORETA solutions” to refer to the 3D EEG source patterns as obtained by the original LORETA software. This procedure reduced inter-subjects variability and was used in previous EEG studies (Babiloni et al., 2004a, in press-a). The general procedure reduced inter-subject variability of the LORETA solutions (Nuwer, 1988). Other methods of normalization using the principal component analysis are effective for estimating the subjective global factor scale of the EEG data (Hernández et al., 1994). These methods are not yet available in the LORETA package, so they were not used in this study.

Solutions of the EEG inverse problem are under-determined and ill conditioned when the number of spatial samples (electrodes) is lower than that of the unknown samples (current density at each voxel). To account for that, the cortical LORETA solutions predicting scalp EEG spectral power density were regularized to estimate distributed rather than punctual EEG source patterns (Pascual-Marqui and Michel, 1994; Pascual-Marqui et al., 1999, 2002). In line with the low spatial resolution of the LORETA technique, we collapsed LORETA solutions at frontal, central, temporal, parietal, occipital, and “limbic” (as defined in the

original LORETA package) regions of the brain model coded into Talairach space. The Brodmann areas listed in Table 2 formed each of these regions of interest (ROIs).

The main advantage of the regional analysis of LORETA solutions was that our modeling could disentangle rhythms of contiguous cortical areas. For example, the rhythms of the occipital source were disentangled with respect to those of the contiguous parietal and temporal sources etc. This was made possible by the fact that LORETA solves the linear inverse problem by taking into account the well-known effects of the head as a volume conductor. With respect to other procedures of data reduction, this type of lobar approach may represent an important reference for multi-modal comparisons with structural and functional neuroimaging methods (SPECT, PET, surface EEG/MEG topography). Finally, it can be stated that the present approach represents a clear methodological improvement compared to surface electrodes EEG spectral analyses. Indeed, the EEG potentials collected at each scalp electrode are strongly affected by head volume conductor effects. For example, occipital electrodes collect scalp potentials generated not only from the occipital cortex but also from parietal and temporal cortices due to head volume conductor effects. In precedence, the LORETA technique including a template head model has been repeatedly used in the investigation of EEG rhythms in physiological and pathological aging (Anderer et al., 2003; Dierks et al., 2000; Huang et al., 2002; Goforth et al., 2004; Babiloni et al., 2004a; Cincotti et al., 2004). This was probably due to the fact that spatial smoothing of the LORETA solutions (resolution in centimeters) and its head template could reliably take into account the slight change in the cortical volume (resolution in millimeters) present in the mild stages of AD.

Statistical analysis of LORETA solutions

STATISTICA 6.0 software was used for the statistical analyses of the normalized regional LORETA solutions. These LORETA solutions relative to AD, MCI, and Nold subjects served as inputs to ANOVA analyses. Subjects’ age, education, and IAF were used as covariates. Mauchly’s test evaluated the sphericity assumption. It evaluates the hypothesis that the sphericity assumption holds (null hypothesis); if the test provides statistically significant values ($P < 0.05$), then the hypothesis of sphericity must be rejected since the assumption is violated (Mauchley, 1940). Correction of the degrees of freedom was made with the Greenhouse–Geisser procedure. Duncan test was used for post hoc comparisons ($P < 0.05$). In particular, two ANOVA designs were used in the present study.

The first ANOVA was a control analysis to verify the sensitivity of the present methodological approach, namely, the existence of

Table 2
Brodmann areas included in the cortical regions of interest (ROIs) of this study

Loreta Brodmann areas into the regions of interest (ROIs)	
Frontal	8, 9, 10, 11, 44, 45, 46, 47
Central	1, 2, 3, 4, 6
Parietal	5, 7, 30, 39, 40, 43
Temporal	20, 21, 22, 37, 38, 41, 42
Occipital	17, 18, 19
Limbic	31, 32, 33, 34, 35, 36

LORETA solutions were collapsed in frontal, central, parietal, temporal, occipital, and limbic ROIs.

differences in the regional normalized LORETA solutions among Nold, MCI, and AD groups. This ANOVA used Group (Nold, MCI, AD), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic) as factors. The existence of those differences would be confirmed by the following two statistical ANOVA results: (i) a statistical ANOVA effect including the factor Group ($P < 0.05$); (ii) a Duncan post hoc test indicating statistically significant differences in the normalized regional LORETA solutions with the pattern $AD \neq Nold$ and MCI (Duncan test, $P < 0.05$). The normalized regional LORETA solutions showing statistically significant pattern $AD \neq Nold$ and MCI were evaluated as linear correlations with the MMSE scores in all subjects as a single group. The linear correlation was computed with the Pearson's test (Bonferroni corrected, $P < 0.05$).

The second ANOVA evaluated the working hypothesis, namely, the existence of differences in the regional normalized LORETA solutions between MCI/AD carriers of the CST3 B haplotype vs. MCI/AD non-carriers of CST3 B. This ANOVA design used Group (MCI, AD), Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic) as factors. The planned Duncan post hoc testing evaluated the prediction of the working hypothesis. The prediction would be confirmed by the following LORETA patterns: CST3 B non-carriers \neq CST3 B carriers in both MCI and AD groups. Of note, the prediction of the working hypothesis was evaluated only in the normalized regional LORETA solutions fitting the pattern $AD \neq Nold$ and MCI as revealed by the previous control ANOVA analysis. It should be stressed that one aim of the present study was the evaluation of the possible differences of the CST3 effects on EEG rhythms in MCI compared to AD groups, namely, a statistical interaction including the factors Group and Genotype. This implied the inclusion of MCI and AD in the same ANOVA design rather than in two separate ANOVA designs.

Results

Control analysis to validate the subject groups

Grand average of the normalized regional LORETA solutions (i.e., normalized voxel spectral density of estimated z -current density) modeling the distributed cortical EEG sources for delta, theta, alpha 1, alpha 2, beta 1, and beta 2 bands presented specific spatial features in Nold, MCI, and AD groups. The Nold group presented alpha 1 LORETA solutions with maximal amplitude values distributed in the parietooccipital regions. Delta, theta, and alpha 2 LORETA solutions had moderate amplitude values when compared to alpha 1 LORETA solutions, especially

in the parietal, temporal, and occipital regions. Finally, beta 1 and beta 2 LORETA solutions were characterized by lowest amplitude values in all cortical regions. Compared to the Nold group, AD patients showed an amplitude increase of delta LORETA solutions, along with a dramatic reduction in amplitude of parietooccipital alpha 1 LORETA solutions. With respect to the Nold and AD groups, MCI subjects showed intermediate amplitude of alpha 1 LORETA solutions and greater amplitude of alpha 2 LORETA solutions.

The characteristics of the recruited Nold, MCI, and AD subjects are shown in Table 3, which summarizes the relevant demographic and clinical data of the participants. The selection of the Nold subjects was made to have a group having similar age, education, and ratios of gender compared to the MCI subjects. Furthermore, we selected a sub-group of AD subjects having similar age, education, and gender compared to the MCI subjects. Four ANOVA analyses using the factor Group (Nold, MCI, AD) were computed to evaluate the presence or absence of statistically significant differences among the Nold, MCI and AD groups for age, education, gender, and MMSE. No statistically significant differences for age ($P > 0.38$), education ($P > 0.35$), and gender ($P > 0.2$) were found. On the contrary, as expected, the ANOVA analysis for the MMSE showed a statistically significant difference ($F(2,200) = 181$; $P < 0.0001$), indicating that the MMSE values were higher in Nold compared to MCI group ($P < 0.000009$) and in MCI compared to AD group ($P < 0.000009$).

The normalized regional LORETA solutions of the Nold, MCI, and AD groups were used as an input to a control ANOVA analysis, to test the hypothesis that the MCI and AD subjects had typical EEG features as described by the literature (see Introduction). Normalized regional LORETA solutions showed a statistical ANOVA interaction ($F(50,5000) = 1.8$; $MSe = 0.8$; $P < 0.0004$) among the factors Group (Nold, MCI, AD), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic). According to the aim of this control analysis, the planned Duncan post hoc testing evaluated the statistically significant differences of the normalized regional LORETA solutions in line with the pattern $AD \neq Nold$ and MCI. That LORETA pattern was fitted by the 5 regional LORETA solutions relative to occipital delta ($P < 0.04$), parietal alpha 1 ($P < 0.02$), occipital alpha 1 ($P < 0.00008$), temporal alpha 1 ($P < 0.007$), and occipital alpha 2 ($P < 0.001$).

The amplitude values of the mentioned 5 LORETA solutions were correlated with the MMSE scores in all subjects as a single group (Pearson's test; threshold $P < 0.01$ to obtain the Bonferroni corrected $P < 0.05$). The MMSE score negatively correlated with the LORETA solutions relative to occipital delta ($r = -0.18$, $P = 0.008$). On the contrary, the MMSE score positively correlated with the LORETA solutions relative to parietal ($r = 0.17$, $P = 0.01$) and

Table 3

Demographic and neuropsychological data of MCI and AD subjects, each sub-divided in two genetic sub-groups: MCI/AD carriers of the CST3 B haplotype and MCI/AD non-carriers of the CST3 B haplotype

N	Nold 74	MCI 84	AD 45
Age (years)	70.1 (± 0.9 SE) 59 to 85 years	70.2 (± 0.8 SE) 53 to 87 years	72.0 (± 1 SE) 54 to 83 years
Gender (M/F)	29/45 (39% M)	33/51 (39% M)	17/28 (38% M)
MMSE	28.4 (± 0.1 SE) 26.2 to 30	25.8 (± 0.3 SE) 20.2 to 30	19.7 (± 0.4 SE) 14 to 28.3
Education (years)	7.6 (± 0.3 SE) 3 to 13 years	7.4 (± 0.4 SE) 2 to 18 years	7.2 (± 0.4 SE) 3 to 13 years

occipital ($r = 0.19$, $P = 0.005$) alpha 1 as well with to occipital alpha 2 ($r = 0.22$, $P = 0.002$). These results confirmed the sensitivity of the methodological approach, namely, the existence of clear differences in the normalized regional LORETA solutions among Nold, MCI, and AD groups.

Topography of EEG cortical sources estimated by LORETA in CST3 B carriers and non-carriers

For illustrative purposes, Fig. 1 maps grand average of the LORETA solutions (i.e., relative z-current density at cortical voxels) modeling the distributed EEG sources for delta, theta, alpha 1, alpha 2, beta 1, and beta 2 bands in MCI/AD subjects not carrying the CST3 B haplotype and in MCI/AD carrying the CST3 B haplotype. In both MCI and AD groups, the CST3 B non-carriers presented alpha 1 LORETA solutions with maximal amplitude values in the parietooccipital regions. Delta, theta, and alpha 2 sources had moderate amplitude values when compared to alpha 1 LORETA solutions. Finally, beta 1 and beta 2 LORETA solutions were characterized by the lowest amplitude values. Compared to

CST3 B non-carriers, CST3 B carriers showed an amplitude reduction of the alpha 1 and alpha 2 LORETA solutions and an amplitude increase of the delta LORETA solutions.

Statistical analysis of EEG cortical sources (LORETA) characterizing CST3 B carriers with respect to CST3 B non-carriers

Before the ANOVA analysis for the evaluation of the working hypothesis, the Kolmogorov–Smirnov test was used to evaluate the Gaussian distribution of the normalized regional LORETA solutions in the MCI and AD subjects, each sub-divided in the two genetic sub-groups: the MCI/AD carriers of the CST3 B haplotype and the MCI/AD non-carriers of the CST3 B haplotype. The results showed that almost all normalized regional LORETA solutions presented a Gaussian distribution in the MCI/AD CST3 B carriers and in the MCI/AD CST3 B non-carriers ($P > 0.1$). The only violations of the Gaussianity were observed for the LORETA solutions relative to occipital beta 1 (in AD CST3 non-carriers), central beta 2 (in MCI CST3 carriers and in MCI/AD CST3 non-

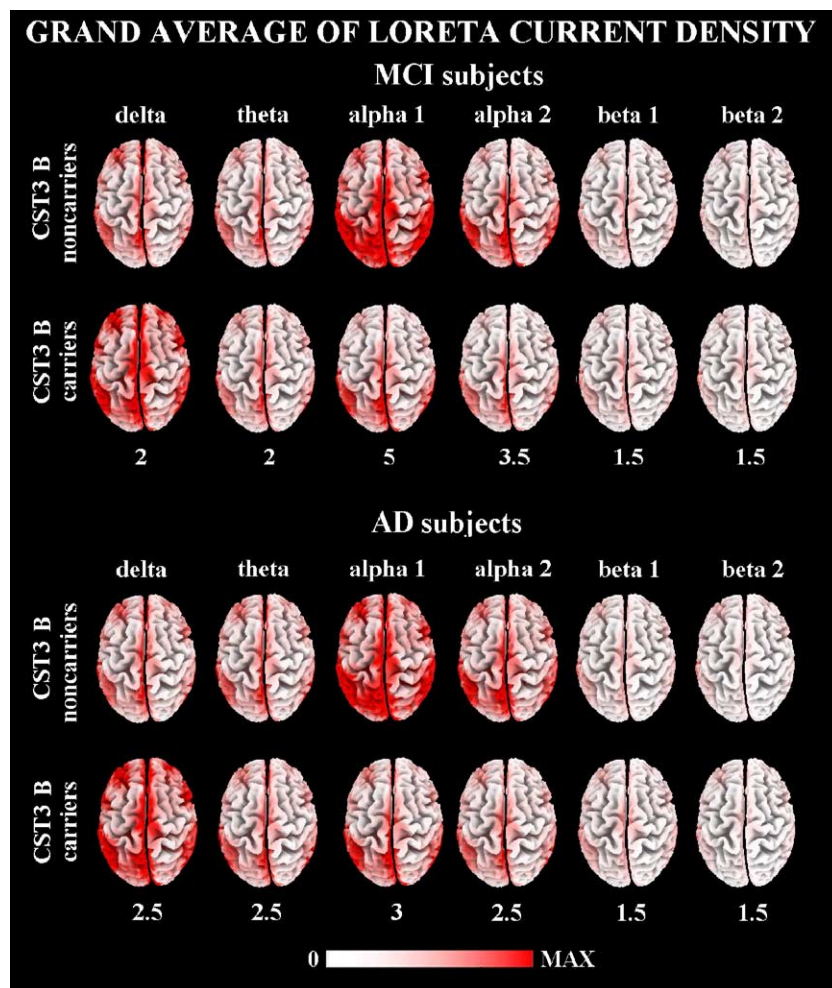


Fig. 1. Grand average of LORETA solutions (i.e., normalized relative current density at the cortical voxels) modeling the distributed EEG sources for delta (2–4 Hz), theta (4–8 Hz), alpha 1 (8–10.5 Hz), alpha 2 (10.5–13 Hz), beta 1 (13–20 Hz), and beta 2 (20–30 Hz) bands in MCI and AD groups, both sub-divided in two genetic sub-groups: MCI/AD carriers of the CST3 B haplotype (high genetic risk for AD) and MCI/AD non-carriers of the CST3 B haplotype. The left side of the maps (top view) corresponds to the left hemisphere. Legend: LORETA, low-resolution brain electromagnetic tomography. Color scale: all power estimates were scaled based on the averaged maximum value (i.e., alpha 1 power value of occipital region in MCI not carrying the CST3 B haplotype). The maximum power value is reported under each column.

carriers), and occipital beta 2 (in MCI CST3 B non-carriers) ($P < 0.05$). These LORETA solutions were not further considered for the ANOVA analysis.

The ANOVA analysis for the evaluation of the working hypothesis showed a statistical interaction ($F(25,3625) = 1.7$; $MSe = 0.6$; $P < 0.02$) among the factors Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic). Fig. 2 shows the normalized regional LORETA solutions relative to this statistical ANOVA interaction. In the figure, the normalized regional LORETA solutions had the shape of EEG relative power spectra. Notably, profile and amplitude of these spectra differed in the diverse cortical regions, thus supporting the idea that scalp EEG rhythms are generated by a distributed pattern of EEG cortical sources.

According to the working hypothesis, the planned Duncan post hoc testing assessed the differences of the normalized regional LORETA solutions between CST3 B carriers and non-carriers: namely, CST3 B carriers \neq CST3 B non-carriers. Of note, the present LORETA pattern CST3 B carriers \neq CST3 B non-carriers was evaluated only in the 5 normalized regional LORETA solutions fitting the pattern AD \neq Nold and MCI, as revealed by the previous control ANOVA analysis. Namely, the LORETA solutions in question referred to occipital delta, parietal alpha 1, occipital alpha 1, temporal alpha 1, and occipital alpha 2. The LORETA pattern CST3 B carriers \neq CST3 B non-carriers was

fitted ($P < 0.01$ threshold for the Bonferroni corrected $P < 0.05$) by the 4 LORETA solutions relative to parietal alpha 1 ($P < 0.000004$), occipital alpha 1 ($P < 0.000003$), temporal alpha 1 ($P < 0.00001$), and occipital alpha 2 ($P < 0.003$). Whereas the LORETA solutions relative to occipital delta showed only a statistical trend ($P < 0.03$ with $P < 0.01$ as a threshold). This was true for both MCI and AD subjects, since there was no interaction between the factors Subject and Genotype.

The ANOVA analysis also showed a statistical interaction ($F(25,3625) = 2.3$; $MSe = 0.6$; $P < 0.0001$) among the factors Group (MCI, AD), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic). Fig. 3 shows mean normalized regional LORETA solutions relative to this statistical ANOVA interaction. The planned Duncan post hoc testing assessed the differences of the normalized regional LORETA solutions between MCI and AD in the 5 normalized regional LORETA solutions fitting the pattern AD \neq Nold and MCI, as revealed by the previous control ANOVA analysis. The LORETA pattern MCI \neq AD was fitted by 4 out of these 5 normalized regional LORETA solutions ($P < 0.01$ for the Bonferroni corrected $P < 0.05$), namely, those relative to occipital delta ($P < 0.002$), occipital alpha 1 ($P < 0.000009$), temporal alpha 1 ($P < 0.0004$), and occipital alpha 2 ($P < 0.000002$). Whereas the LORETA solutions relative to parietal alpha 1 showed only a statistical trend ($P < 0.02$ with $P < 0.01$ as a threshold).

STATISTICAL ANOVA INTERACTION BETWEEN GENOTYPE, BAND AND ROI

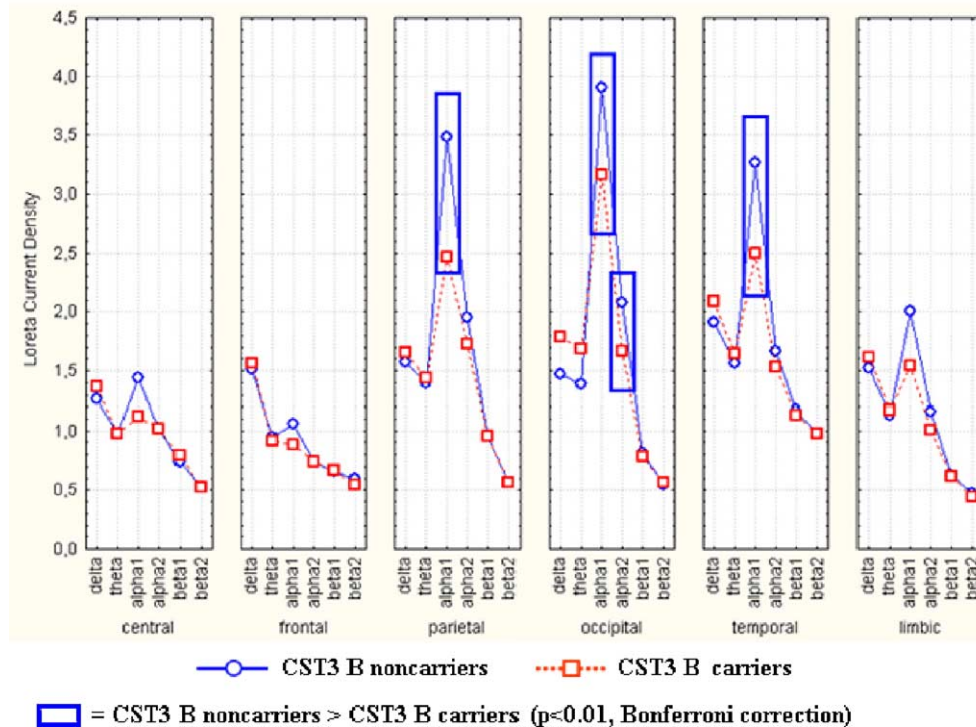


Fig. 2. Normalized regional LORETA solutions (mean across subjects) relative to a statistical ANOVA interaction among the factors Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, “limbic”). This ANOVA design used the normalized regional LORETA solutions as a dependent variable. Subjects’ age, education, and individual alpha frequency peak (IAF) were used as covariates. Normalized regional LORETA solutions modeled the EEG relative power spectra as revealed by a sort of “virtual” intracranial macro-electrodes located on the macrocortical regions of interest. Legend: the rectangles indicate the cortical regions and frequency bands in which normalized regional LORETA solutions presented statistically significant different values between subjects not carrying the CST3 B haplotype and CST3 B carriers ($P < 0.05$). See Methods for further details.

STATISTICAL ANOVA INTERACTION BETWEEN GROUP, BAND AND ROI

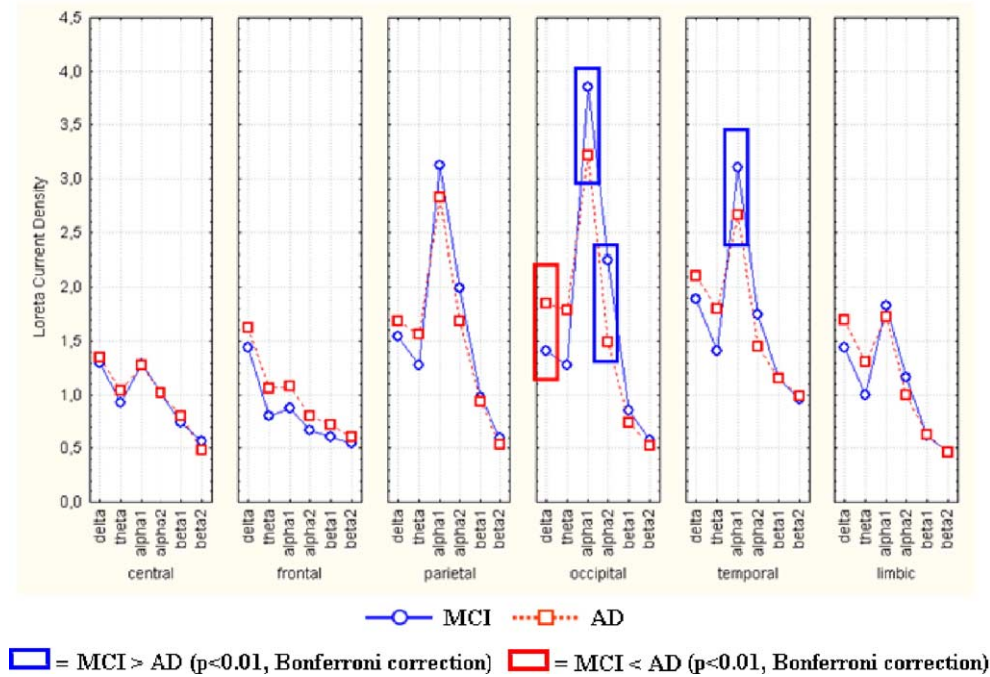


Fig. 3. Normalized regional LORETA solutions (mean across subjects) relative to a statistical ANOVA interaction among the factors Group (MCI, AD), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic). This ANOVA design used the normalized regional LORETA solutions as a dependent variable. Subjects' age, education, and individual alpha frequency peak (IAF) were used as covariates. Legend: the rectangles indicate the cortical regions and frequency bands in which LORETA solutions presented statistically significant different values between MCI and AD groups ($P < 0.05$). See Methods for further details.

Control analyses

A first control ANOVA analysis was carried out to assure that the above-described LORETA source differences due to the factor Genotype (presence or absence of CST3 B) were not affected by a possible interaction between CST3 and ApoE $\epsilon 4$ (even if the number of ApoE $\epsilon 4$ carriers were paired in the groups to be compared). We considered sub-groups of MCI ($N = 52$) and mild AD ($N = 24$) subjects, having no ApoE $\epsilon 4$ carriers and practically equal age, education, and ratios of gender. Table 4 reports the means of personal and neurophysiological parameters of these sub-groups. The LORETA solutions were used as a dependent variable. The ANOVA design was the same using all subjects and groups including paired ApoE $\epsilon 4$ carriers. There was a statistical interaction ($F(25,1800) = 5.35$; $MSe = 0.56$; $P < 0.0001$) among the factors Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central,

frontal, parietal, occipital, temporal, limbic), fully confirming the results of the ANOVA design including all subjects (higher delta and lower alpha amplitude in CST3 B carriers than non-carriers regardless the patients' group type). Fig. 4 shows the mean regional LORETA solutions relative to that statistical ANOVA interaction.

A second control ANOVA analysis was carried out to confirm that the differences in the LORETA solutions between CST3 B carriers and non-carriers were not due to differences in the IAF. In the control ANOVA analysis, we considered three EEG sub-bands, whose band limits were defined according to the IAF (Klimesch, 1996, 1999; Klimesch et al., 1998). The frequencies of the three sub-bands were (i) from IAF $- 4$ Hz to IAF $- 2$ Hz, (ii) from IAF $- 2$ Hz to IAF, and (iii) from IAF to IAF $+ 2$ Hz. The normalized regional LORETA solutions at these three sub-bands were used as a dependent variable. The ANOVA factors were Group (MCI, AD), Genotype (presence or absence of CST3 B), Sub-band (IAF $- 4$ to

Table 4

Demographic and neuropsychological data of MCI and AD subjects, each sub-divided in two genetic sub-groups: MCI/AD carriers of the CST3 B haplotype and MCI/AD non-carriers of the CST3 B haplotype

	MCI		AD	
	CST3 AA	CST3 AB	CST3 AA	CST3 AB
<i>N</i>	26	26	12	12
Age (years)	69.6 (± 1.3 SE)	69.6 (± 1.9 SE)	75.8 (± 1.3 SE)	75.8 (± 2.1 SE)
Gender (M/F)	10/16	10/16	4/8	4/8
MMSE	25.9 (± 0.5 SE)	25.9 (± 0.5 SE)	20.4 (± 0.7 SE)	20.5 (± 1.3 SE)
Education (years)	7.1 (± 0.7 SE)	7.1 (± 0.9 SE)	7.3 (± 1 SE)	7.1 (± 1.5 SE)

The number of MCI subjects was 26 for each genetic sub-group, having no ApoE $\epsilon 4$ carriers and practically equal age, education, and ratios of gender. Similarly, the number of AD subjects was 12 for each genetic sub-group, having no ApoE $\epsilon 4$ carriers and practically equal age, education, and ratios of gender.

STATISTICAL ANOVA INTERACTION BETWEEN GENOTYPE, BAND, ROI

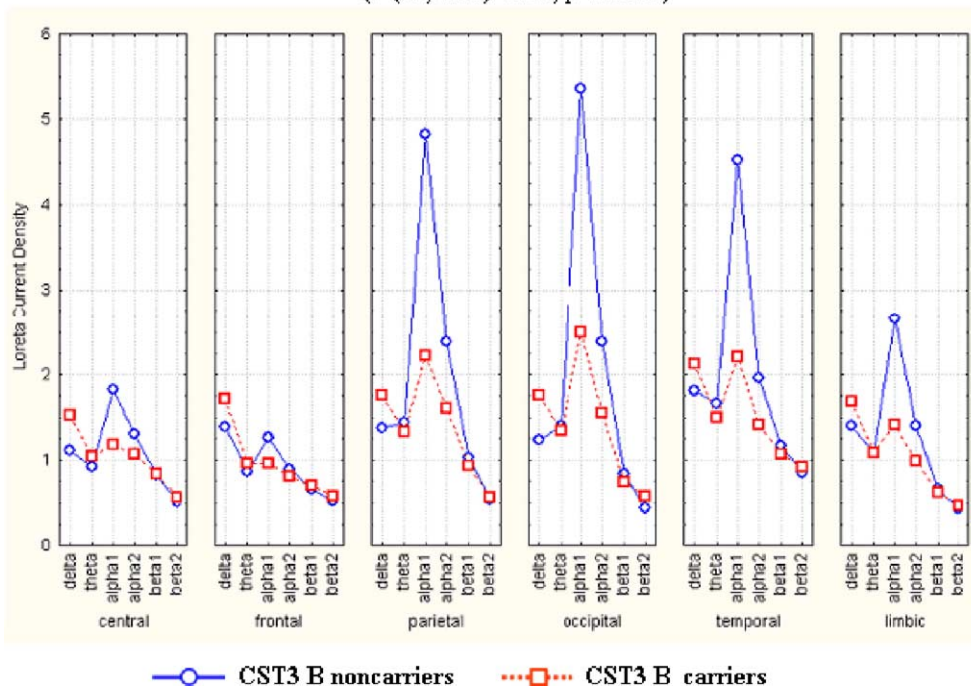
(F (25,1800)=5.35, $p < 0.0001$)

Fig. 4. Regional LORETA solutions (mean across subjects) relative to a statistical ANOVA interaction among the factors Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal, limbic). This ANOVA design used the normalized relative current density values at ROI level as a dependent variable. Subjects' age, education, and individual alpha frequency peak (IAF) were used as covariates. The number of MCI subjects was 26 for each genetic sub-group, having no ApoE $\epsilon 4$ carriers and practically equal age, education, and ratios of gender. Similarly, the number of AD subjects was 12 for each sub-group, having no ApoE $\epsilon 4$ carriers and practically equal age, education, and ratios of gender.

IAF $- 2$, IAF $- 2$ to IAF, IAF to IAF $+ 2$), and ROI (central, frontal, parietal, occipital, temporal, “limbic”). Subjects' age and education were used as covariates. There was a statistical interaction ($F(10,1450) = 1.8$; $MSe = 0.85$; $P < 0.05$) among the factors Genotype, Sub-band, and ROI. Fig. 5 shows the mean normalized regional LORETA solutions relative to this statistical ANOVA interaction. The normalized regional LORETA solutions showed lower amplitude in CST3 carriers than in CST3 non-carriers at the sub-band from IAF $- 2$ Hz to IAF Hz (parietal and temporal areas, $P < 0.01$) and at the sub-band from IAF Hz to IAF $+ 2$ Hz (parietal, occipital and temporal areas, $P < 0.001$). Furthermore, there was a statistical interaction ($F(10,1450) = 1.8$; $MSe = 0.85$; $P < 0.05$) among the factors Group, Sub-band, and ROI. The normalized regional LORETA solutions showed higher amplitude in MCI than in AD group at the sub-band from IAF $- 2$ Hz to IAF Hz (occipital area, $P < 0.0004$) and at the sub-band from IAF Hz to IAF $+ 2$ Hz (occipital area, $P < 0.0001$). On the whole, these ANOVA results fully confirmed those obtained with the fixed EEG bands.

To cross-validate the LORETA results on CST3 B non-carriers and CST3 B carriers, the statistical ANOVA analysis was directly repeated on the EEG data used as an input for the LORETA analysis. The same frequency bands of interest of the LORETA analysis were considered, namely, delta (2–4 Hz), theta (4–8 Hz), alpha 1 (8–10.5 Hz), alpha 2 (10.5–13 Hz), beta 1 (13–20 Hz), and beta 2 (20–30 Hz). Five ROIs were considered. These ROIs included, respectively, (i) C3, Cz, and C4 electrodes for the central region, (ii) F3, Fz, and F4 electrodes for the frontal region, (iii) P3, Pz, and P4 electrodes for the parietal region, (iv) O1, O2 electrodes for the occipital region, (v) T3, T4, T5, T6 for the temporal region.

Compared to the LORETA results, here the “limbic” region was excluded due to its deep location. The same kind of normalization of the LORETA solutions was used for the EEG spectral solutions. The spectral power density at each electrode was normalized to the spectral power density averaged across all frequencies (0.5–45 Hz) and across all electrodes. The values of normalized spectral power density of the electrodes belonging to the same ROI were averaged at each of the 6 frequency bands of interest. The values of the normalized, regional spectral power density served as a dependent variable of the ANOVA analysis. The ANOVA factors (levels) were Group (MCI, AD), Genotype (presence or absence of CST3 B), Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2), and ROI (central, frontal, parietal, occipital, temporal). Subjects' age and education were used as covariates. This ANOVA design pointed to a statistical interaction ($F(5,725) = 3.45$; $MSe = 6.39$; $P < 0.004$) between factors Genotype and Band (see Fig. 6). Duncan post hoc testing showed that, regardless the ROI factor, alpha 1 spectral density power was stronger in amplitude in the CST3 B non-carriers compared to CST3 B carriers ($P < 0.002$). Furthermore, regardless the ROI factor, the delta spectral density power pointed to a statistical trend ($P < 0.07$), showing lower amplitude in the CST3 B non-carriers compared to CST3 B carriers. Finally, the alpha 2 spectral density power was slightly stronger in amplitude in the CST3 B non-carriers compared to CST3 B carriers. On the whole, these ANOVA results confirmed in terms of frequency bands (delta, alpha 1) the differences among CST3 B carriers and CST3 B non-carriers in line with the results of the LORETA analysis. As expected, in the control analysis, the statistical effects were spatially blurred on all regions of the scalp (namely, there was

STATISTICAL ANOVA INTERACTION BETWEEN GENOTYPE, SUB-BAND AND ROI

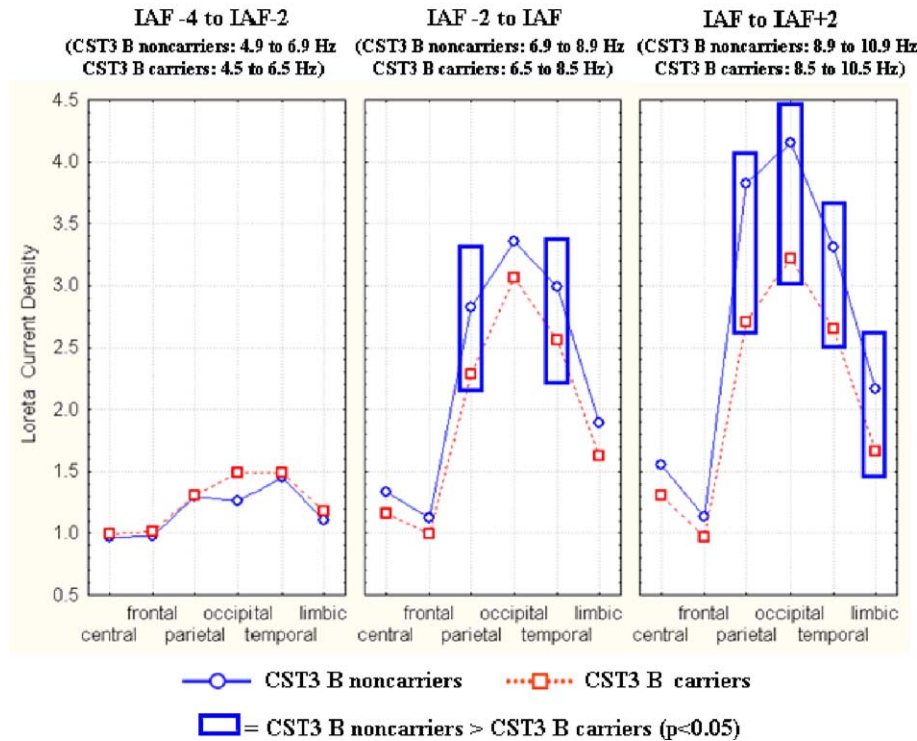


Fig. 5. Normalized regional LORETA solutions (mean across subjects) relative to a statistical ANOVA interaction among the factors Genotype (presence or absence of CST3 B), Sub-band according to IAF (IAF -4 to IAF -2 , IAF -2 to IAF, IAF to IAF $+2$), and ROI (central, frontal, parietal, occipital, temporal, “limbic”). The normalized regional LORETA solutions of these three EEG sub-bands were used as a dependent variable. Subjects’ age and education were used as covariates. Legend: the rectangles indicate the cortical regions and frequency bands in which the normalized LORETA solutions presented statistically significant differences between CST3B non-carriers and CST3 B carriers ($P < 0.05$).

no statistical interaction with the factor ROI). The low spatial resolution of the standard EEG technique was also unable to detect the statistical effect only localized at occipital alpha 2 source by the LORETA analysis.

Discussion

In the present study, the control analysis (i.e., resting EEG, normalized regional LORETA solutions) allowed the modeling of regional cortical EEG sources in Nold, MCI, and mild AD subjects posed at resting condition. With reference to the Nold and MCI subjects, the AD subjects were characterized by a significant amplitude decrease of the alpha 1 sources (parietal, occipital, and temporal areas) and of the alpha 2 sources (occipital areas). Furthermore, in AD subjects, there was a significant magnitude increase in the occipital delta sources. The amplitude of occipital delta, parietal alpha 1, occipital alpha 1, and occipital alpha 2 sources showed linear correlations with MMSE score (global cognitive level) across all Nold, MCI, and mild AD subjects considered as a single group. These results are in line with previous evidence showing an increase of slow rhythms in MCI and AD than Nold subjects (Grunwald et al., 2001; Jelic et al., 2000; Wolf et al., 2003; Babiloni et al., 2004a) and a decrease of alpha rhythms in MCI than Nold subjects (Dierks et al., 1993; 2000; Jelic et al., 1996, 2000; Rodriguez et al., 1999a,b; Huang et al., 2000; Grunwald et al., 2001; Frodl et al., 2002; Babiloni et al., 2004a;

Moretti et al., 2004). Therefore, the present results validated both the general EEG methodological approach and the procedure for the selection of MCI and AD subjects, as a preliminary basis for the original observations on the relationships between CST3 genotype and brain rhythmicity.

EEG characteristics in MCI and AD subjects carrying CST3 B haplotype

The most striking result of the present study was that CST3 B genetic risk factor for AD similarly influences EEG rhythmicity in both MCI and AD subjects. Interestingly, alpha 1 sources in parietal, occipital, and temporal areas showed lower amplitude in CST3 B carriers than non-carriers ($P < 0.00001$). Furthermore, occipital alpha 2 sources showed lower amplitudes in CST3 B carriers than non-carriers ($P < 0.003$). Conversely, occipital delta sources unveiled stronger amplitude in CST3 B carriers than non-carriers (statistical trend, $P < 0.03$). These results are fully in agreement with recent evidence showing a relationship between CST3 B haplotype and late-onset AD, independently of ApoE factor (Crawford et al., 2000; Finckh et al., 2000; Beyer et al., 2001; Olson et al., 2002; Lin et al., 2003; Goddard et al., 2004). Furthermore, they complement those obtained by studying the relationship between ApoE $\epsilon 4$ genetic risk and cortical EEG rhythmicity in AD subjects (Lehtovirta et al., 1995, 1996), especially at alpha rhythms (Jelic et al., 1997; Lehtovirta et al., 2000). Of note, the present findings cannot be explained by invoking the effects of psychoactive drugs, ApoE $\epsilon 4$ or

STATISTICAL ANOVA INTERACTION BEWEN GENOTYPE AND BAND

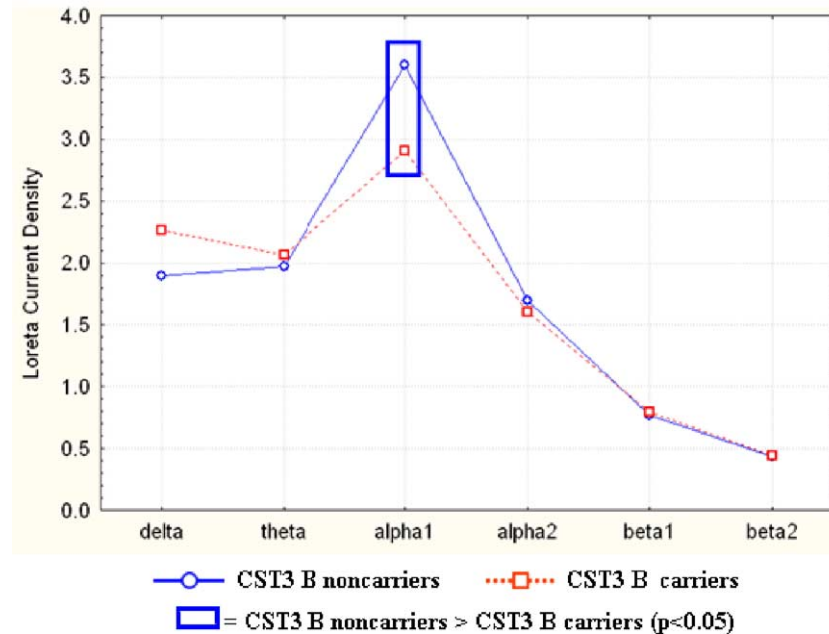


Fig. 6. Normalized regional EEG spectral power density relative to a statistical ANOVA interaction among the factors Genotype (presence or absence of CST3 B) and Band (delta, theta, alpha 1, alpha 2, beta 1, beta 2). This control ANOVA design was focused on the EEG data used as an input for the LORETA analysis, in order to cross-validate the LORETA solutions. The ANOVA design used the normalized regional EEG spectral power density as a dependent variable. Subjects' age, education, and individual alpha frequency peak (IAF) were used as covariates. Legend: the rectangles indicate the scalp regions and frequency bands in which normalized regional EEG spectral power density presented statistically significant different values between CST3 B carriers and CST3 B non-carriers ($P < 0.05$). See Methods for further details.

co-morbidity in our MCI and AD subjects with CST3 B haplotype (see Methods).

The results of the present study warrant further EEG investigations in normal subjects who carry the CST B haplotype. Here, we were unable to do it, since many of them did not give consent to the blood sample extraction. Future investigations should evaluate whether and to what extent the CST3 B effects on EEG rhythms in MCI and AD subjects may be confounded with (or induced by) clinical symptoms development. Indeed, previous evidence has shown a reduction of the resting posterior cortical activity in cognitively intact individuals with ApoE $\epsilon 4$ allele, compared to subjects not carrying it (Small et al., 1995; Reiman et al., 1996). In parallel to other genetic AD risk factors (e.g., homocysteine; Anello et al., 2004; Malaguarnera et al., 2004), ApoE and CST3 might impinge independently upon the process of beta amyloid deposition even in clinically normal carriers. In these individuals, environmental risk factors (diet, vitamins, pro-oxidants, metals, hormones, toxins, cognitive stimulations, etc.) might unveil flaws due to genetic risk factors. At the present stage of research, we do not know the level of interdependence between CST3 and ApoE risk factors and cannot exclude possible interactions between ApoE and CST3 with respect to their EEG effects and amyloid load. These are very important issues for future research. Here, we could demonstrate the "pathological" effects of CST3 B on EEG rhythms not only when the number of ApoE $\epsilon 4$ carriers between the groups was paired but also when these carriers were removed from the statistical analysis.

A crucial question of the present study is why sources of delta and alpha rhythms were modulated in amplitude in MCI and AD subjects with CST3 B haplotype. It can be speculated that the

physiological mechanism is related to the protective role of cystatin C in neuronal functioning and survival (Lofberg and Grubb, 1979; Ohe et al., 1996; Taupin et al., 2000; Palmer et al., 2001; Huh et al., 1999; Palm et al., 1995; Ishimaru et al., 1996; Miyake et al., 1996; Katakai et al., 1997). Impaired neuroprotection of cystatin C in CST3 B carriers (Benussi et al., 2003) might indirectly influence thalamocortical and cortico-cortical (mainly cholinergic) systems that produce delta and alpha rhythms (Sarter and Bruno, 2002; Kobayashi and Tadashi, 2002; Muzur et al., 2002). These systems display different spontaneous rhythms dependent on the behavioral state of vigilance. Corticofugal volleys are effective in synchronizing slow (<15 Hz) and fast (20–50 Hz) rhythms in thalamocortical networks during sleep and awake conditions, respectively (Steriade, 1997). During slow wave sleep, corticofugal slow oscillations (<1 Hz) are effective in grouping thalamic-generated spindles (7–14 Hz) and delta (1–4 Hz) rhythms and in hyperpolarizing forebrain cholinergic neurons (Steriade, 2003). In the case of brain arousal, spindles and delta rhythms are blocked by inhibition of oscillations generated by cortico-thalamic (14 Hz), and thalamocortical (1–4 Hz) neurons. These rhythms are then replaced by fast (beta and gamma) cortical oscillations induced by the depolarizing effects of mesopontine cholinergic neurons acting on thalamocortical neurons and by the depolarizing effects of nucleus basalis cholinergic neurons acting on cortical neurons (Steriade, 2003).

Keeping in mind the mentioned theoretical framework and neuroprotective effects of Cystatine C, it can be speculated that the increment of delta oscillations in MCI and AD subjects carrying CST3 B is related to loss of hippocampal and posterior

cortical neurons, which are impinged by cholinergic inputs. Several lines of evidence provide support to that speculation. It has been shown that early degeneration in mesial temporal cortex of MCI and AD subjects can affect functional connectivity between hippocampal formation and temporoparietal cortex (Killiany et al., 1993). Furthermore, a bilateral reduction of gray matter volume in the hippocampal formation and entorhinal cortex of AD subjects was correlated with an increment of delta rhythms in posterior cortex (Fernandez et al., 2003). Finally, increase of slow EEG rhythms in AD was related to progressive cortical hypoperfusion of blood typically associated with neuronal loss (Kwa et al., 1993; Steriade, 1994; Passero et al., 1995; Nobili et al., 1998; Rodriguez et al., 1999a). However, the speculative nature of the above explanation needs an extensive experimental confirmation by future investigations.

In the present study, the major EEG changes in MCI and AD subjects carrying the CST B haplotype were observed at low-band alpha rhythms. From a physiological point of view, wakeful alpha rhythms are mainly modulated by thalamocortical and cortico-cortical interactions (Steriade and Llinas, 1988; Brunia, 1999; Pfurtscheller and Lopez da Silva, 1999). Low-band alpha would be mainly related to subject's global attentional readiness, whereas high-band alpha would reflect the engagement of specific neural channels for the elaboration of sensorimotor or semantic information (Rossini et al., 1991; Steriade and Llinas, 1988; Klimesch, 1996; Klimesch et al., 1997, 1998). At rest condition, the voltage of the alpha rhythms would be inversely correlated with the cortical excitability and with the level of attentional processes depending on the novelty and importance of the stimulus. For this reason, it has been suggested that the amplitude of alpha rhythms and corresponding cortical excitability reflect time-varying inputs of forebrain cholinergic pathways (Ricceri et al., 2004). In this theoretical framework, a reasonable explanation is that, in MCI and AD subjects carrying CST3 B, a reduction of Cystatine C impairs the functioning of forebrain cholinergic neurons impinging upon thalamic and cortical generators of alpha rhythms. Indeed, we think that the effect is related to "functioning" rather than "neuronal loss," since previous evidence has not shown a clear relationship between alpha rhythms and atrophy of mesial temporal and posterior cortical areas in AD subjects (Fernandez et al., 2003). The present explanation should be the matter of future EEG investigations manipulating cholinergic agonists and antagonists in subjects carrying and not carrying CST3 B haplotype.

Conclusions

The present study tested the hypothesis that cortical sources of resting EEG rhythms are more impaired in MCI or AD carriers of the CST3 B haplotype than in non-carriers. Indeed, the source amplitude of alpha 1 (parietal, occipital, temporal areas) and alpha 2 (occipital area) was statistically lower in CST3 B carriers than non-carriers ($P < 0.01$). Furthermore, there was a statistical trend indicating that amplitude of occipital delta sources was stronger in CST3 B carriers than in non-carriers ($P < 0.03$; $P < 0.01$ as a threshold). Thus, for the first time, a significant relationship between CST3 genotype and global neurophysiological phenotype (i.e., cortical EEG rhythms) was demonstrated in MCI and AD subjects. The effects were independent of ApoE $\epsilon 4$ co-presence. However, further studies should explore possible complex interactions among CST3 and other genetics risk factors (e.g., ApoE) in

the modulation of EEG rhythms in physiological and pathological aging. The present findings prompt future studies aiming to the identification of MCI individuals with extremely high statistical chances of progressing to dementia based on combined genetics and EEG examination.

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