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Altered NCAM Expression Associated with the Cholinergic System in Alzheimer's Disease

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Abstract. Neurotransmitter system dysfunction and synapse loss have been recognized as hallmarks of Alzheimer's disease (AD). Our hypothesis is that specific neurochemical populations of neurons might be more vulnerable to degeneration in AD due to particular deficits in synaptic plasticity. We have studied, in postmortem brain tissue, the relationship between levels of synaptic markers (NCAM and BDNF), neurochemical measurements (cholinacetyltransferase activity, serotonin, dopamine, GABA, and glutamate levels), and clinical data (cognitive status measured as MMSE score). NCAM levels in frontal and temporal cortex from AD patients were significantly lower than control patients. Interestingly, these reductions in NCAM levels were associated to an ApoE4 genotype. Levels of BDNF were also significantly reduced in both frontal and temporal regions in AD patients. The ratio between plasticity markers and neurochemical measurements was used to study which of the neurochemical populations was particularly associated to plasticity changes. In both the frontal and temporal cortex, there was a significant reduction in the ChAT/NCAM ratio in AD samples compared to controls. None of the ratios to BDNF were different between control and AD samples. Furthermore, Pearson's product moment showed a significant positive correlation between MMSE score and the ChAT/NCAM ratio in frontal cortex ($n = 19$; $r = 0.526$*; $p = 0.037$) as well as in temporal cortex ($n = 19$; $r = 0.601$*; $p = 0.018$) in AD patients. Altogether, these data suggest a potential involvement of NCAM expressing neurons in the cognitive deficits in AD.

Keywords: BDNF, ChAT, cognitive deficits, frontal cortex (BA10), MMSE, plasticity, temporal cortex (BA20)

INTRODUCTION

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disorder characterized by a gradual loss of cognitive function and is the major form of dementia affecting the elderly. Since the first reports of loss of basal forebrain acetylcholine-synthesizing neurons and associated neurochemical perturbation of presynaptic cholinergic markers [1,2], neurotransmitter system dysfunction and synapse loss have been recognized as hallmarks of AD along with senile plaques and neurofibrillary tangles [3–7]. The cholinergic dysfunction, together with glutamatergic deficits, is considered that may trigger, in part, some of aspects of

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cognitive disturbance in AD [8–11]. Deficiencies in central serotonergic [12–15] and perhaps dopaminergic [16], but see also [17,18] neural transmission may also play a critical role in some of the clinical manifestations of AD. Significant reductions in γ-amino butyric acid (GABA) levels have also been described in cases of AD [19].

Our hypothesis is that specific neurochemical populations of neurons might be more vulnerable to degeneration in AD due to deficits in synaptic plasticity. One class of molecules that may contribute to plasticity in the central nervous system (CNS) are glycoproteins involved in the adhesion of neural cells. These adhesion glycoproteins are believed to regulate the stabilization of synaptic junctions, neurite outgrowth, wound repair, functional plasticity, and control release of neurotransmitter. The neural cell adhesion molecule (NCAM) is a part of a family of cell-surface glycoproteins that plays key roles in normal brain development, including axonal/dendritic growth and branching, and synaptic plasticity [20–23]. Through alternative splicing, three major isoforms of NCAM are generated: NCAM120, -140, and -180. NCAM can be also modified by the addition of α-2,8-polysialic acid (PSA-NCAM). At the systems level, NCAMs have been implicated as critical components in the induction of long-term potentiation (LTP) and in memory formation [20,21] NCAMs have been shown to play critical roles in ontogenetic development and are thus potential candidates in expression of age-related pathology, particularly in AD [24,25].

The aim of the present work was to study if a particular neurochemical deficit is associated to NCAM alterations in AD. We have checked the proportion of the amount of NCAM relative to the amount of each of the neurotransmitters studied [26]. For comparative purposes, we have studied another classically used marker of plasticity, neurotrophin brain-derived neurotrophic factor (BDNF), an activity-dependent secreted protein that is critical to organization of neuronal networks and synaptic plasticity (see recent review [27]). Reduced levels of BDNF have been documented in studies of postmortem brain tissue from AD patients [28–30]. Moreover, BDNF mRNA levels were decreased in AD in the nucleus basalis of Meynert, the main source of cholinergic innervation to the cerebral cortex [31,32].

We have used clinical data and postmortem brains (frontal and temporal cortex), collected as part of a prospective community-based study of dementia [33], to study the relationship between levels of synaptic markers (BDNF and NCAM), neurochemical measurements (cholinacetyltransferase activity, serotonin, dopamine, GABA, and glutamate levels) and clinical data (cognitive status). On the other hand, inheritance of the ε4 allele of the apolipoprotein E gene (ApoE4) is a major risk factor for the development of AD. Although the association between ApoE4 and AD is well documented, the mechanism by which ApoE exerts an isoform-specific effect on neurons in disease is unknown. Therefore, we thought of interest to check the influence of expressing the ApoE allele on synaptic changes in AD. Based on our data, we propose that there is an association between cholinergic alterations, NCAM expression, and ApoE genotype, which might be related to the cognitive deficits in AD.

Table 1
Demographic details

	Control	Alzheimer's disease
Number of patients	16	19
Gender (man/woman)	9/7	11/8
ApoE (2,3/4)	$-$/16	12/7
Braak stage	1–2	5–6
Age (years)	78.75 ± 2.67	81.06 ± 1.60
range	53–99	64–89
Postmortem delay (h)	39.28 ± 5.40	48.63 ± 6.30
pH	6.28 ± 0.16	6.44 ± 0.10

Values are mean $\pm$ S.E.M. pH, standard chemical symbol, negative log of hydrogen ion concentration; S.E.M, Standard error of the mean. Values are mean $\pm$ S.E.M. ApoE4 $=$ AD patients lacking one or two copies of the ApoE4 allele. There were no significant differences between age, postmortem delay, or brain pH in either control patients or those with dementia (Student t-test, $p > 0.05$).

MATERIAL AND METHODS

Patients and assessment of behavior

A total of 35 individuals from the Thomas Willis Oxford Brain Collection were included in the study, 19 patients with clinical diagnosis of dementia, and 16 elderly normal controls matched for age, gender, postmortem delay, and brain pH (Table 1). Those patients with dementia were an autopsied subset of subjects included in a prospective study of behavioral changes in clinically diagnosed as demented patients [33]. Drug histories were recorded for all patients, and none of the patients with AD received cholinomimetics. At entry to the study assessment, diagnoses were made using Cambridge Mental Disorders of the Elderly Examination (CAMDEX) [34], DMS-III-R criteria [35], and NINCDS-ADRA criteria [36]. Cognitive status was assessed using the Mini-Mental State Examination (MMSE) [37]. Severity of dementia was deter-

mined according to MMSE score at last interview before death. All tissues from control patients were examined by a pathologist and were confirmed to be free of gross neuropathology; clinical information indicated no gross neurological or psychiatric disorder.

Tissue samples and neuropathology

For all subjects, informed consent had been obtained from relatives before the removal of brain tissue at death and subsequent use of the material for research. The study had Local Ethics Committees' approval. Selection of subjects for the study was based on tissue availability, not gender, age, or disease severity. At autopsy, brains were removed and blocks corresponding to frontal (Brodmann area 10, BA10) and temporal (Brodmann area 20, BA20) cortex and were stored at $-80\,^{\circ}$C until processing. All patients were found to meet CERAD criteria [4] for a diagnosis of AD, and all brains were Braak stage 5 or 6 as assessed by a neuropathologist. To partially mitigate the possible effects of cause of death on neurochemical determinations, brain pH was measured with deionised water [38] as an index of acidosis associated with terminal coma (Table 1). Brain pH is used as an indication of tissue quality in postmortem research, with pH $>$ 6.1 considered acceptable [39]. All subsequent analyses were performed blind to clinical information.

Cholinacetyltransferase (*ChAT*) *activity*

ChAT activity was performed as described [14]. Frontal or temporal cortex was homogenized in 50 vol. of 0.87 mM EDTA containing 0.1% Triton X-100 (pH 7.0). Duplicated samples of 10 μl from homogenates were used in the assay. The reaction mixture comprised the following: 87 nM EDTA (pH 7.4), 0.5 M NaH_2PO_4 (pH 7.4), 40 mM choline chloride 3 M NaCl, 2 nM eserine salicylate, and 2 mM [^{14}C]-actetyl CoA (Amersham, UK). Samples were incubated at 37 °C for 30 min. Water was used as sample blanks. The reaction was terminated by adding 100 μl of cold water. The acetylcholine product was separated using Kalignost solution: 0.5% cold di-sodiumtetraphenylborate in 15–85% acetonitrile-toluene. The supernatant was then transferred to scintillation cocktail (Ecoscint TM) and radioactivity was measured. Results were expressed as percentage of control values.

Serotonin, dopamine, glutamate, and GABA measurements

Concentrations of different neurotransmitters were determined by high performance liquid chromatography (HPLC) with electrochemical detection (Waters Spheribor® S10 0DS2 4,6 $\times$ 150 mm), including precolumn derivatization with o-phthaldehyde and β-mercapthoethanol [40] for GABA and glutamate determinations.

As previously described [14], for serotonin (5-HT) and dopamine (DA) determinations, tissue was homogenized in 20 v of extraction mixture (0.4 M percloric acid; 1 mM EDTA; 0.1% metabisulphitic acid). Homogenates were centrifugated 32,500 g for 20 min. The mobile phase consisted of 80:16 (v/v) mixture of buffer ($KH_2PO_4{\cdot}2H_2O$ 0.1 M, citric acid 0.1 M, EDTA 1 mM and octanosulphonic acid 0.74 mM; pH $=$ 3). 5-HT content was calculated by comparing with a 1 ng standard. The limit of detection was 1 pg/10 μl. DA content was calculated by comparing with a 0.5 ng standard. The limit of detection was 1 pg/10 μl.

For GABA or glutamate determinations, tissue was homogenized in 200 v of 0.1 M di-sodiumtetraborate (pH 9.1). Homogenates were centrifuged at 32,500 g for 20 min. The mobile phase consisted of 72:28 (v/v) mixture of buffer (NaH_2PO_4 0.1 M, pH $=$ 5.5) and methanol; the mixture was filtered and degassed through a 0.22 μm nitrocellulose membrane (Millipore, UK). Glutamate and GABA content were calculated by comparing with a 2 ng standard. The limit of detection was 20 pg/10 μl for glutamate and 50 pg/10 μl for GABA content.

Quantification of total levels of BDNF

The samples were processed with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) measuring BDNF protein (BDNF Emax immunoassay system, Promega Co, USA). All samples and standards were prepared in duplicate. Incubation and washing were conducted according to the manufacturer's instructions with reagents from the kit, at room temperature, sealed, and on a shaker. The optical density of the wells was analyzed in a Bio-Rad Microplate Reader at 450 nm. A standard curve was generated from the serial BDNF standard dilutions, and BDNF protein concentrations in the samples were extrapolated directly from the standard curve. BDNF levels were expressed as pg/mL.

Quantification of total levels of NCAM

Crude synaptosomal pellets were obtained according to a protocol from [41]. Hippocampal tissue was homogenized in 10 vol of ice-cold sucrose (0.32 M) and HEPES (5 mM) buffer that contained a cocktail of protease inhibitors (Complete TM, Boehringer Mannheim, UK) and centrifuged at 1000 g for 5 min. The supernatant was then centrifuged again at 15,000 g for 15 min, and the pellet resuspended in Krebs buffer. Protein concentration for each sample was estimated as previous [42]. NCAM levels were quantified according to a previously described protocol [25]. Flat bottom 96-well microplates were allowed to adsorb a coating solution (Na_2CO_3 0.1 M/ $NaHCO_3$, 0.1 M) for 2 h at room temperature. The solution was removed and 50 μl of pellet samples added at a concentration of 10 mg/ml to each well of polystyrene flat-bottom ELISA plates. Plates were incubated overnight at 4°C and then washed three times with 1 M phosphate buffered saline (PBS) containing 0.05% Tween 20, pH 7.4. Additional binding sites were blocked with bovine serum albumin (BSA) (3%) for 2 h at room temperature. Wells were incubated with 50 μl aliquots of primary antibody Ab5032 (1:15,000 TBST; Chemicon) for 20–24 h at 4°C and subsequently, 50 μl aliquots of antirabbit IgG peroxidase conjugate antibody (1:500; Sigma, UK) were added for a 2 h incubation period. TMB (Promega) was used as a chromogenic substrate. The reaction was terminated by the addition of 1 N hydrochloric acid. Absorbancies were measured at 450 nm using an automatic ELISA microplate reader. Results were expressed as absorbance.

ApoE genotyping

ApoE genotyping was performed with DNA extracted from tissues according to [43].

Statistical analysis

Data were analyzed by SPSS for Windows, release 15.0. Normality was checked by Shapiro-Wilks's test ($p > 0.05$). Student's t-test was used in comparisons between controls and AD samples. The effects of demographic factors (age, postmortem delay, and brain pH) on neurochemical variables, intercorrelation between neurochemical variables or relationships between MMSE score and neurochemical measures were determined by Pearson's or Spearman's correlation coefficients, according to the normality of variables.

RESULTS

There were no significant differences in age, postmortem delay, or brain pH between the control and AD groups. A summary of demographic details of controls and AD patients is shown in Table 1. In addition, no significant correlations between age, postmortem delay, or brain pH and any of the neurochemical variables studied in either controls or AD ($p > 0.05$) were found. However, in a conservative analysis of data, they were included as covariates in order to avoid age or postmortem delay interferences in subsequent analysis.

Neurochemical characterization of samples

As shown in Table 2, ChAT activity in AD was reduced by 70% (BA10) or 60% (BA20) in comparison to control. Significant decreases in 5-HT, DA, and GABA levels were also found both in BA10 or BA20 samples from AD patients. Glutamate content in AD brains was not significantly different from that observed in controls.

Plasticity markers in Alzheimer's disease

NCAM levels in frontal and temporal cortex from AD patients were significantly lower than control patients (Student's t-test; $p < 0.05$). Reductions reached 22% (1.00 ± 0.10 vs. 0.79 ± 0.04 pg/ml) in BA10 and 21.7% (0.51 ± 0.04 vs. 0.40 ± 0.02 pg/ml) in BA20 when compared to control values (Fig. 1A). None of the controls showed an ApoE4 genotype. Interestingly, these reductions in NCAM levels in AD were associated to an ApoE4 genotype: significant reductions were found in patients showing an ApoE 4 genotype (control vs. AD, in pg/ml, BA10: 1.00 ± 0.10 vs. 0.71 ± 0.07; BA20: 0.51 ± 0.04 vs. 0.36 ± 0.02), while no differences in NCAM levels were found in AD patients lacking one or two copies of the ApoE4 allele (control vs. AD, in pg/ml, BA10: 1.00 ± 0.10 vs. 0.93 ± 0.05; BA20: 0.51 ± 0.04 vs. 0.48 ± 0.03).

BDNF levels were significantly reduced (Student's t-test; $p < 0.05$) in both frontal and temporal regions in AD patients. BDNF reductions reached 40% (2123.21 ± 223.57 vs. 1277.6044 ± 251.24 pg/ml) and 47% (2603.35 ± 364.53 vs. 1439.33 ± 163.18 pg/ml) in BA10 and BA20 regions respectively (Fig. 1B). No effects of ApoE genotype on BDNF reductions in AD patients were found.

Table 2
Neurochemical measurements and their ratio to plasticity markers in the frontal (BA10) and temporal (BA20) cortex of controls and Alzheimer's disease (AD) patients

	Frontal cortex (BA 10)		Temporal cortex (BA20)	
	Control	AD	Control	AD
ChAT	100.00 ±9.82	27.68 ± 3.55*	100.00 ± 7.34	38.82 ± 7.88*
ChAT/NCAM	108.15 ± 12.52	41.28 ± 7.64*	214.17 ± 16.79	89.08 ± 21.12*
ChAT/BDNF	0.06 ± 0.12	0.05 ± 0.02	0.05 ± 0.02	0.04 ± 0.01
5-HT	42.95 ± 3.25	25.41 ± 1.99*	44.50 ± 2.86	23.95 ± 1.39*
5-HT/NCAM	53.66 ± 7.96	37.75 ± 5.82	92.92 ± 9.37	62.62 ± 3.91*
5-HT/BDNF	0.03 ± 0.05	0.05 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
DA	91.70 ± 15.51	43.55 ± 5.84*	109.35 ± 16.77	45.45 ± 7.07*
DA/NCAM	106.63 ± 27.05	56.42 ± 9.66	187.32 ± 37.03	110.56 ± 19.63
DA/BDNF	0.04 ± 0.01	0.09 ± 0.03	2.79 ± 0.55	4.63 ± 0.55
GABA	704.39 ± 63.41	555.33 ± 31.97*	579.94 ± 31.72	457.44 ± 31.70*
GABA/NCAM	901.06 ± 144.66	791.44 ± 100.67	1237.57 ± 129.40	1256.17 ± 108.19
GABA/BDNF	0.47 ± 0.11	0.70 ± 0.23	0.31 ± 0.05	0.45 ± 0.09
Glutamate	5767.11 ± 411.73	5297.91 ± 322.52	4967.06 ± 233.81	4986.11 ± 357.35
Glutamate/NCAM	7117.01 ± 1032.51	7385.94 ± 1013.41	10150.54 ± 825.25	12990.99 ± 1105.91
Glutamate/BDNF	3.67 ± 0.75	11.48 ± 4.15	0.05 ± 0.01	0.04 ± 0.01

Values are mean ± S.E.M from control ($n = 16$) and Alzheimer's disease patients ($n = 19$). ChAT activity is expressed as percentage of activity relative to control. 5-HT, dopamine (DA), GABA and glutamate levels are expressed as pg/mg of tissue. *Significantly lower than Control, Student t-test, $p < 0.01$ or $p < 0.001$.

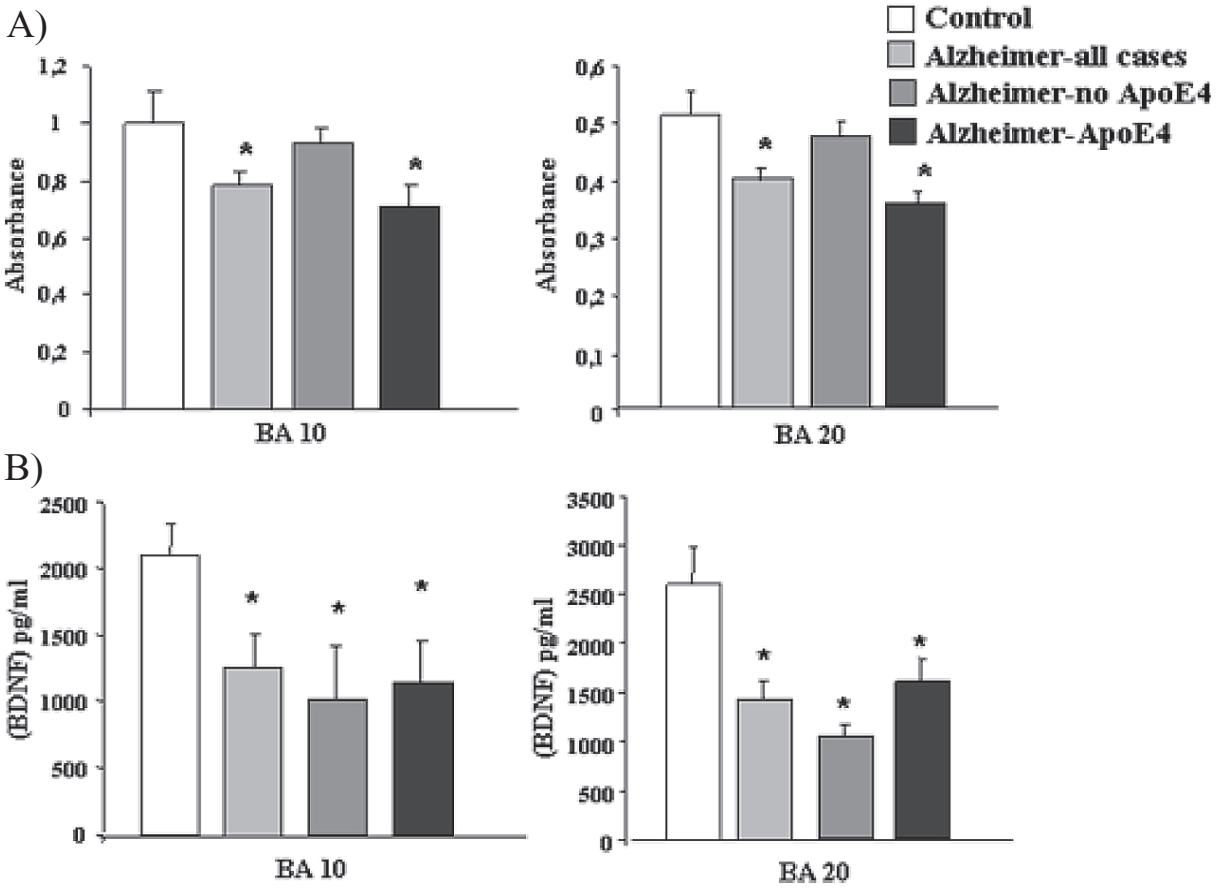

Fig. 1. Reductions in plasticity markers in the frontal (BA10) and temporal (BA20) cortex of Alzheimer's disease (AD) patients. A) NCAM levels, expressed as absorbance; B) BDNF levels. $N = 16$–19; $^*p < 0.05$ vs. control, Student's t-test.

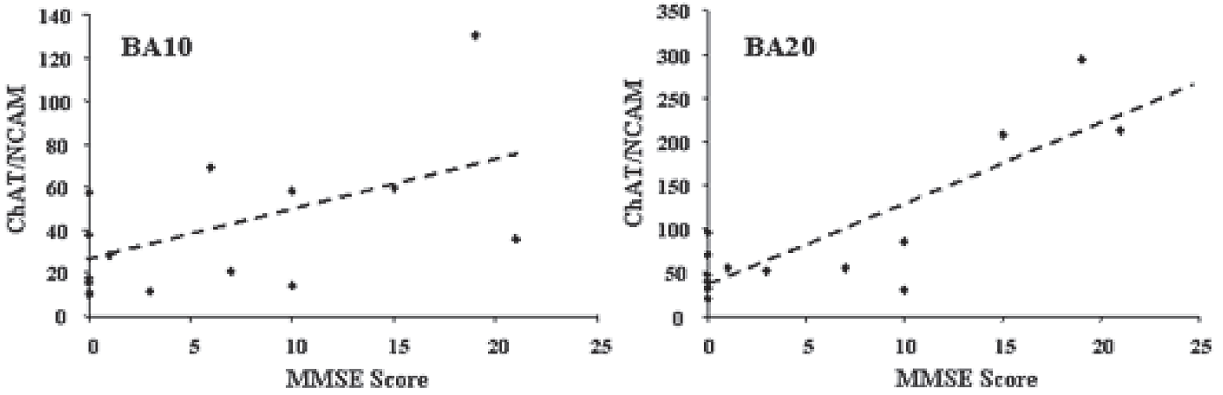

Fig. 2. Significant correlations (Pearson's correlation coefficient) between ChAT/NCAM ratio and MMSE score in the frontal (BA10, $n = 19$, $r = 0.526$, $p = 0.037$) and temporal cortex (BA20, $n = 19$, $r = 0.601$, $p = 0.018$) in Alzheimer's disease (AD) patients.

Relationship between plasticity markers and neurochemical measurements

The ratio between plasticity markers and neurochemical measurements was used to study which of the neurochemical populations was particularly associated to plasticity changes. In the frontal cortex, there was a significant reduction (Student's t-test; $p < 0.05$ or $p < 0.01$) in ChAT/NCAM and 5-HT/NCAM ratios in AD samples compared to controls. None of the ratios to BDNF were different between control and AD samples. In the temporal cortex, only the ratio ChAT/NCAM was significantly lower in AD samples. All these results are shown in Table 2.

Correlations with cognitive status

In AD patients mean MMSE score before death was 5 ± 1 (range varies between 0 and 21).

ChAT activity correlated to MMSE score both in the frontal (Pearson's product moment, $n = 19$; $r = 0.524$*; $p = 0.018$) and temporal cortex ($n = 19$; $r = 0.759$*; $p < 0.001$). NCAM levels did not correlate to MMSE score ($n = 19$; $r = 0.150$; $p = 0.564$ for the frontal cortex, $n = 19$; $r = 0.142$; $p = 0.599$ for the temporal cortex). Statistical analysis showed a significant positive correlation between MMSE score and the ChAT/NCAM ratio in frontal cortex ($n = 19$; $r = 0.526$*; $p = 0.037$) as well as in temporal cortex ($n = 19$; $r = 0.601$*; $p = 0.018$) in AD patients (Fig. 2). When split by ApoE genotype, these correlations were associated to an ApoE4 genotype ($n = 7$; $r = 0.796$*; $p = 0.032$ for the frontal cortex, $n = 7$; $r = 0.526$*; $p = 0.037$ for the temporal cortex), while no correlations were found in AD patients lacking the ApoE4 genotype ($n = 12$; $r = 0.211$; $p = 0.789$ for the frontal cortex, $n = 12$; $r = 0.938$; $p = 0.062$ for the temporal cortex). BDNF levels or BDNF ratios did not correlate to MMSE score in either cortical region. No other correlation related to other neurotransmitter systems has been found.

DISCUSSION

In the present work, we have found that: 1) NCAM levels are decreased in the frontal and temporal cortex of AD patients, and these decreases are associated with an ApoE4 genotype; and 2) the ratio of ChAT/NCAM is significantly reduced in the frontal and temporal cortex of AD patients. Furthermore, the ChAT/NCAM ratio, both in the frontal and temporal cortex, and particularly in ApoE4 carriers, was correlated to cognitive deficits in AD. Altogether, these data suggest a potential interaction between the cholinergic system and NCAM expressing neurons, which functionally correlates to cognitive deficits.

Synaptic loss is the major neurobiological substrate of cognitive dysfunction in AD. Synaptic failure is an early event in the pathogenesis that is clearly detectable already in patients with mild cognitive impairment, a prodromal state of AD (reviewed by [44]). It progresses during the course of AD and in most early stages involves mechanisms of compensation (synaptic remodeling or synaptogenesis) before reaching a stage of decompensated function (degeneration) [45,46]. This synaptic degeneration in AD leads to important neurochemical alterations in the brains of AD patients and different neurotransmitter systems have been shown to be altered in AD (see review [47]). The neurochemical

correlates of clinical dysfunctions in AD are not yet fully understood [48], but changes in neurochemistry could be the key to understanding the pathogenesis of cognitive deficits. Among them, the classical hallmark of AD is the disruption of basal forebrain cholinergic pathways and consequent cholinergic denervation of the neocortex and hippocampus. This cholinergic dysfunction has been largely related to disturbance of attentional aspects of cognition [10,11,49].

In experimental studies, NCAM expression seems to be important, and perhaps essential, for long-term information storage [50,51]. For example, administration of NCAM antibodies impaired spatial learning and passive avoidance learning in rats [52]. Administration of a synthetic peptide, which mimics NCAM activation, enhanced spatial memory [53]. Furthermore, null mutation of the NCAM gene in mice leads to impaired spatial learning and memory [54,55]. At a molecular level, expression of LTP was suppressed by the administration of antibodies against NCAM or synthetic peptides interfering with NCAM activity [56–58]. A recent study, [59] postulated that NCAM was upregulated during the early stages of spatial water maze learning, supporting the implication of this NCAM regulatory mechanism on spatial learning and memory.

No changes [60] or increased [61,45] levels of NCAM have been reported in the hippocampus of AD patients, but this increase has been related to increased neurogenesis, and may be indicative of an attempt of the brain to restore structure and function or to compensate for the damage caused by the disease. Soluble forms of NCAM are increased in the cerebrospinal fluid of both AD and Parkinson disease patients, but these increases seem to be related to aging and neurodegeneration and not to dementia as such [62]. In the serum samples, only levels of low molecular weight forms of NCAM correlated to severity of dementia [63]. When focusing on the cortex, according to our results, decreased levels of NCAM have been found in the frontal cortex of AD patients [64], probably reflecting synaptic loss in the illness. An interesting finding of the present work is the association of NCAM decreases in AD to an ApoE4 genotype. Inheritance of the $\varepsilon 4$ allele of the ApoE gene (ApoE4) is a major risk factor for the development of AD. Although the association between ApoE4 and AD is well documented, the mechanism by which ApoE exerts an isoform-specific effect on neurons in disease is little known. On top of already proposed mechanisms, we suggest that the presence of the ApoE4 allele might differential affect NCAM-expressing neurons [65], since neither BDNF levels (present data) nor synaptophysin or PSD95 levels [66] were affected by the ApoE4 genotype. However, it has to be noted that none of the controls showed an ApoE4 genotype. The lack of information of the effect of ApoE4 in controls prevents us to discriminate if the NCAM reduction is due to AD in ApoE4 carriers or just to the effect of the E4 isoform in general. It is known that ApoE4 carriers (with and without neurodegenerative disorders) have less synaptic plasticity and regeneration processes than E3 carriers [67]. On the other hand, the enhanced synaptic regenerative processes in non-carriers of the ApoE4 allele could lead to an enhanced NCAM synthesis/expression and therefore, could be masking NCAM decreases associated to AD.

As expected, considering that most of the patients in this study were severe AD (MMSE score equal or less than 10), decreases in most of the neurochemical/plasticity markers studied have been found. The ratio between plasticity markers and neurochemical measurements was used to study which of the neurochemical populations was associated with changes in NCAM and/or BDNF. Our results seem to suggest that there is a selective loss of cholinergic neurons expressing NCAM in AD. As an alternative explanation, it can be also argued that the ratio ChAT/NCAM decreases because decreases in NCAM levels in AD are smaller that decreases in ChAT activity. In any case, this relationship between ChAT-NCAM seems to be related to cognitive alterations in AD, as suggested by present data on the correlation between ChAT/NCAM ratio and MMSE score. It is noticeable that considering the components of the ratio, only ChAT levels correlated *per se* with cognitive status. Therefore it seems that loss of cholinergic neurons expressing NCAM, and particularly in ApoE4 carriers, has driven the statistical correlation. It has to be considered that even though the functional link between cholinergic neurons and NCAM-expressing neurons has been described in the periphery, little is known regarding co-expression of cholinergic-NCAM neurons in the CNS. In light of the present data, future studies, e.g., using immunohistochemical methods, could confirm our hypothesis of a selective loss of ChAT labeling in NCAM expressing neurons. As to the mechanism responsible for the association of ChAT-NCAM, it is of note that in the same cohort of postmortem samples used for the present study, the presence of the ApoE4 allele selectively correlated to loss of ChAT activity [68].

In addition, for comparative purposes, we have studied BDNF, another classically used marker of plasticity. It is widely accepted that alterations of BDNF levels

or its receptors occur during normal aging and AD (see review, [32]). The lack of significant changes in the ratio to BDNF in any of the neurochemical markers used seem to support our hypothesis that synaptic changes in cholinergic neurons seem to be specifically related to NCAM expressing terminals. A note of caution should be mentioned at this point, pointing to the interest for future studies of measuring NCAM level, as well as the rest of the molecules implicated in cognition, in AD in early stages, when cognitive impairment exists but the brain is less affected by numerous pathological changes.

The present data support the notion that treatments to increase or mimic the function of NCAM might improve neuronal connectivity and cognition, perhaps through a cholinergic-mediated mechanism. NCAM-mimicking peptides are being developed for their potential efficacy [69,70], although the therapeutic efficacy of these NCAM-peptide mimetics remains to be tested. In a recent study the ability of C3d, a NCAM mimetic peptide, to promote ChAT activity in septal cholinergic neurons was reported [71]. On the other hand, treatment with cholinesterase inhibitors, which improve cholinergic function, increases PSA-NCAM in the hippocampus [72]. Furthermore, these enhancements correlated directly with improved spatial learning ability, suggesting that an improved memory-associated synaptic plasticity may be the fundamental mechanism underlying the disease modifying action of this kind of drugs [73]. In this sense, pharmacological manipulation of the cholinergic system may improve not only cognitive function but also plasticity disturbances in dementia.

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