Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease

(choline acetyltransferase/nicotinic receptor/muscarinic receptor/tacrine)

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ABSTRACT   Apolipoprotein E (apoE) is critical in the modulation of cholesterol and phospholipid transport between cells of different types. Human apoE is a polymorphic protein with three common alleles, $APO\varepsilon2$, $APO\varepsilon3$, and $APO\varepsilon4$. ApoE4 is associated with sporadic and late-onset familial Alzheimer disease (AD). Gene dose was shown to have an effect on risk of developing AD, age of onset, accumulation of senile plaques in the brain, and reduction of choline acetyltransferase (ChAT) activity in the hippocampus of AD subjects. To characterize the possible impact of the apoE4 allele on cholinergic markers in AD, we examined the effect of apoE4 allele copy number on pre- and postsynaptic markers of cholinergic activity. ApoE4 allele copy number showed an inverse relationship with residual brain ChAT activity and nicotinic receptor binding sites in both the hippocampal formation and the temporal cortex of AD subjects. AD cases lacking the apoE4 allele showed ChAT activities close or within age-matched normal control values. The effect of the apoE4 allele on cholinomimetic drug responsiveness was assessed next in a group ($n = 40$) of AD patients who completed a double-blind, 30-week clinical trial of the cholinesterase inhibitor tacrine. Results showed that >80% of apoE4-negative AD patients showed marked improvement after 30 weeks as measured by the AD assessment scale (ADAS), whereas 60% of apoE4 carriers had ADAS scores that were worse compared to baseline. These results strongly support the concept that apoE4 plays a crucial role in the cholinergic dysfunction associated with AD and may be a prognostic indicator of poor response to therapy with acetylcholinesterase inhibitors in AD patients.

Apolipoprotein E (apoE), a 34-kDa protein, mediates the binding of lipoproteins to the low density lipoprotein receptor. It functions as a ligand in receptor-mediated internalization of lipid-rich lipoproteins and is also involved in reverse lipid transport (1). In the central nervous system (CNS), apoE plays a key role in mobilization and redistribution of cholesterol and phospholipid during membrane remodeling associated with synaptic plasticity (2–4). The importance of apoE to brain lipid transport is underscored further by the absence of other key plasma apolipoproteins such as apoA1 and apoB (5) in this tissue. ApoE mRNA (3) and protein (6) are found predominantly in astrocytes within the CNS. The apoE gene on chromosome 19 has three common alleles ($APO\varepsilon2$, $-\varepsilon3$, and $-\varepsilon4$), which encode three major apoE isoforms. Recently, the frequency of the $apo\varepsilon4$ allele was shown to be markedly increased in sporadic (7–9) and late-onset familial Alzheimer disease (AD) (10, 11). Most interestingly, a gene dosage effect was observed in both familial (10) and sporadic (7) cases—

namely, as age of onset increases, $APO\varepsilon4$ allele copy number decreases. Women show increased $APO\varepsilon4$ allele frequency when compared to age-matched men (7). The association between apoE4 and AD, however, appears to vary across ethnic groups (9). The presence of apoE4 has been shown to modulate the age of onset in AD families with the amyloid precusor protein mutation (12) but not in families with chromosome 14-linked early-onset AD (13).

Previous studies have shown that apoE mRNA levels are slightly increased (14) or unchanged (15) in postmortem brains of AD patients. Recently, apoE protein concentration was shown to be reduced in the cortex and hippocampus of $APO\varepsilon4$ AD subjects (16). AD subjects with the $APO\varepsilon4/\varepsilon4$ genotype showed half the hippocampal apoE concentration measured in $APO\varepsilon3/\varepsilon3$ AD or control subjects. $APO\varepsilon4$ allele copy number also has an impact on the risk of developing AD on the accumulation of mature senile plaques in the cortex and hippocampus (for a review, see ref. 17).

Brain membrane phospholipids, especially phosphatidylcholine (PC) and phosphatidylethanolamine (PE), have been shown to be involved in the availability of choline, a rate-limiting precursor of acetylcholine (ACh) (18). The release from PC of free choline precursor for ACh synthesis is accomplished in a one-step process through a phospholipase D-type enzyme in cholinergic neurons. Brain levels of choline are decreased by up to 40–50% in frontal and parietal cortices (19) of AD patients, whereas cholesterol, which is required for the proper functioning of certain cholinergic receptor subtypes (20), was shown to be markedly reduced in AD vs. control subjects (21). It was recently proposed that the low apoE concentrations reported in the brains of $APO\varepsilon4$ AD subjects could compromise cholesterol and phospholipid transport in the CNS and may selectively damage the cholinergic system, which relies heavily on lipid homeostasis (17). As losses of cholinergic neurons and/or choline acetyltransferase (ChAT) activity are well known neurochemical hallmarks of AD (22, 23), the relationship between the apoE4 genotype and cholinergic deficits is highly relevant to investigate in genetically distinct individuals. Preliminary studies indicate that ChAT activity is markedly reduced in the hippocampus (17) and cortex (24, 25) of apoE4 AD subjects. The major aims of the present study were thus (i) to investigate the apparent status

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer disease; apoE, apolipoprotein E; ChAT, choline acetyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CNS, central nervous system; ADAS-Cog, AD assessment scale, cognitive component; MMSE, mini-mental state evaluation; CIBIC, clinician interview-based impression of change; IADL, instrumental activities of daily living scale; PSMS, physical self-maintenance scale; PDS, progressive deterioration scale; GDS, global deterioration scale.
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and integrity of the cholinergic system in AD and control brain subjects with different apoE genotypes and (*ii*) to establish whether the therapeutic response to a cholinomimetic treatment such as the acetylcholinesterase (AChE) inhibitor tacrine in AD subjects is dependent on the *APOε4* allele copy numbers.

MATERIALS AND METHODS

Case Selection and ApoE Genotyping. Frozen tissues from 24 autopsy-confirmed cases of sporadic AD (9 females, 15 males; 72 ± 1 years old) and from 19 control individuals (9 females, 10 males; 72 ± 2 years old) were obtained from the Douglas Hospital Brain Bank in Montréal, Canada. Neuropathological criteria pertaining to these brain samples have been described (26, 27). The average postmortem delay was 14 ± 1 and 12 ± 2 hr for AD and control subjects, respectively. Duration of the disease was 8 ± 2 years for apoE4-negative subjects vs. 10 ± 3 years for apoE4 carriers. It should be noted that the availability of *APOε4/ε4* homozygous subjects is very limited due to the fact that *APOε4/ε4* homozygous individuals represent <2% of the normal population. ApoE genotype was determined by allele-specific extension of purified brain DNA as described (28).

Cholinergic Markers. Markers examined in this study include ChAT activity, N-[^{3}H]methylcarbamoylcholine nicotinic binding sites, [^{3}H]pirenzepine (PZ) muscarinic M1 sites, and [^{3}H]AF-DX 116 muscarinic M2 sites. Protocol pertaining to these assays has been described in detail by Aubert *et al.* (27). Cholinergic cell density in the medial basal forebrain was assessed as described by Etienne *et al.* (26). Briefly, two 30-μm-thick sections were sampled at 500-μm intervals starting at the rostral edge of the anterior commissure; one served to reveal the AChE pattern by the procedure of Karnowski and Roots (29) with ethopropazine (0.1 mM) to assess specificity, and the other was stained with cresyl violet. In Ch2 (vertical limb of the nucleus basalis of Meynert/diagonal band of Broca) and Ch4 (nucleus basalis of Meynert), all neurons larger than 30 μm with a visible nucleus and abundant Nissl substance were counted as described (26). Mapping studies in human nucleus basalis of Meynert have shown that all large cells fitting those criteria are indeed cholinergic (30).

Clinical Evaluation. Patients were selected from the group completing the entire 30-week tacrine trial who showed maximal worsening or improvement on the AD assessment scale, cognitive component (ADAS-Cog). Demographic variables regarding the subjects enrolled in this clinical study can be found in Table 1. All patients received the maximum dose of 160 mg/day. The ADAS-Cog is a 70-point scale where decline in scores indicates improvement. Patients were also assessed by the ADAS, the mini-mental state evaluation (MMSE), the clinician interview-based impression of change (CIBIC), the instrumental activities of daily living scale (IADL), the physical self-maintenance scale (PSMS), the progressive deterioration scale (PDS), and the global deterioration scale (GDS)

Table 1. Demographic variables for tacrine-treated subjects

	Non-E4 ($n = 18$)	E4 ($n = 22$)	P value*
Education			
Elementary	2.5%	10.0%	
Secondary	22.5%	25.0%	0.33
College	20.0%	20.0%	
Gender			
Male	30.0%	27.5%	0.29
Female	15.0%	27.5%	
Age, years	74	73	0.95

*Interaction between apoE genotype and demographics (*t* test).

(31, 32). Differences were judged between baseline and 30-week evaluations. Genotype determination for apoE was then performed as described (7) and cognitive performance was contrasted with the presence or absence of the apoE4 allele.

RESULTS AND DISCUSSION

Fig. 1 illustrates alterations in various cholinergic markers as a function of *APOε4* allele dosage in postmortem brains of AD subjects. A marked *APOε4* allele-dependent reduction of ChAT activity and nicotinic binding sites was observed in the hippocampus and temporal cortex of AD subjects. In contrast, muscarinic binding sites (M1 and M2) were relatively spared, irrespective of the apoE genotype. These results clearly suggest that apoE4 compromises some cholinergic markers in AD in a gene-dependent manner. The fact that both ChAT activity and nicotinic receptor sites are altered by the presence of the *APOε4* allele is of special interest as ChAT is a well known marker of presynaptic cholinergic terminals and a portion of nicotinic receptors is also believed to be presynaptically localized (27), possibly explaining their losses in AD.

To determine whether the reduction was due to specific cholinergic nerve terminal alterations or a likely consequence of the loss of cholinergic neurons originating from the basal forebrain, previously published cholinergic cell counts in the nucleus basalis of Meynert (26) were reexamined in postmortem subjects for which apoE genotypes were determined by allele-specific PCR amplification.

Fig. 2 illustrates the effect of *APOε4* allele dose on AChE-positive neuron density in the nucleus basalis of Meynert (Ch4a and Ch4i) and diagonal band of Broca (Ch2) in a group of AD and control subjects. As reported before (26), AChE-positive neuronal density is reduced in AD vs. control subjects. Genotype stratification reveals that apoE4 AD carriers show a more pronounced reduction in cell density in the Ch2 and Ch4 areas when compared to apoE4-negative AD subjects. Nucleolar volume is not altered in remaining AChE-positive cells, whether apoE4 is present or not. Taken together, these results clearly indicate that AChE-positive cholinergic neurons of the basal forebrain/cortical–hippocampal projections are particularly at risk in apoE4 carriers.

ChAT activities in the hippocampus and cortex were shown to be rather similar in control and AD subjects *not* carrying the *APOε4* allele, despite a marked loss (−60%) of AChE-positive neurons in the basal forebrain. This is consistent with compensatory remodeling of the cholinergic projections reported to occur in a small group of AD subjects (33). On the other hand, comparative losses of basal forebrain AChE-positive neurons in apoE4 carriers were accompanied by a marked reduction of ChAT activity and nicotinic binding sites in terminal projection areas, suggesting a potentially impaired synaptic plasticity in these subjects.

Recently, Masliah and colleagues (34) demonstrated that apoE knockout mice fail to show synaptic plasticity in response to entorhinal cortex lesions when compared to nonknockout littermates. Cortical synaptic density is markedly reduced in 18-month-old knockout mice (34). Furthermore, cognitive performance in the Morris–Swimmaze test of 2-month-old apoE knockout mice was shown to be significantly impaired when compared to normal mice (35). This observation is consistent with the recent findings that apoE concentrations are lower in the cerebrospinal fluid of AD subjects compared to normal subjects (36) and that *APOε4* carriers showed marked reductions in apoE levels in the hippocampus and cortex compared to normal controls and *APOε3/ε3* AD subjects (16).

These results suggest that reduction of apoE levels in the CNS may result in marked alterations of synaptic integrity and plasticity in rodents as well as in humans. The fact that cholesterol is normally transported by apoE-containing li-
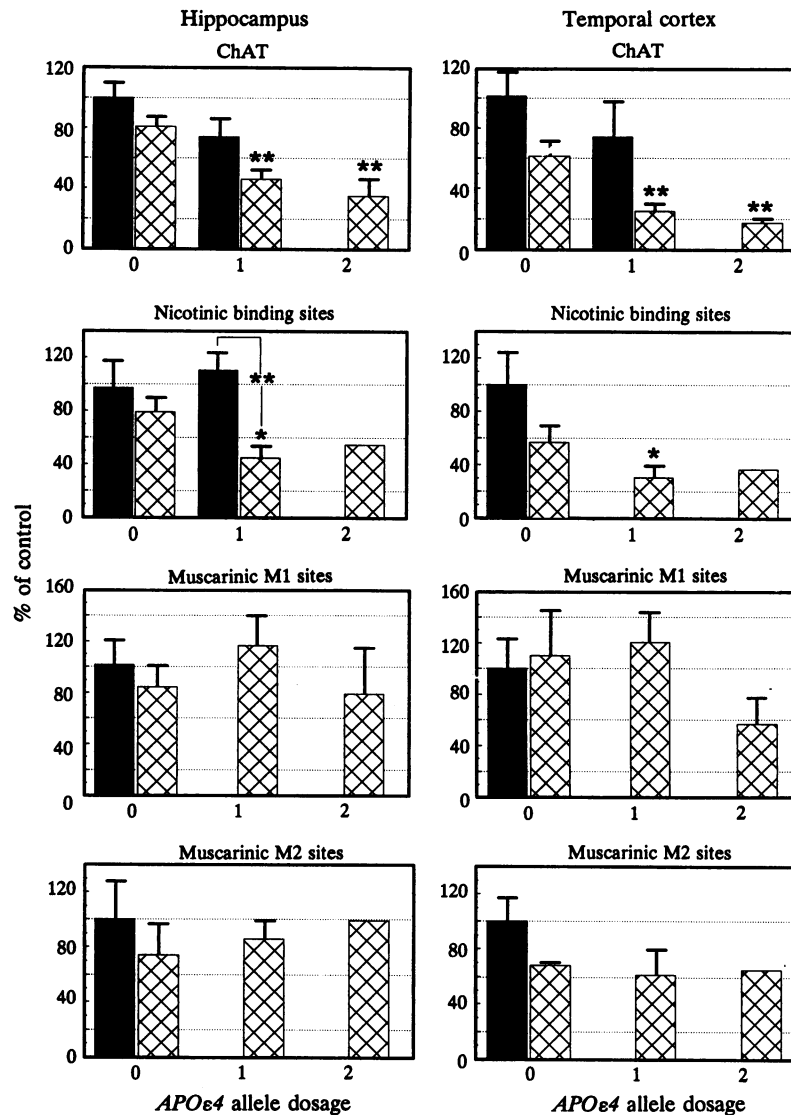
FIG. 1.   ChAT activity, *N*-[³H]methylcarbamoylcholine nicotinic binding sites, [³H]pirenzepine muscarinic M1, and [³H]AF-DX 116 muscarinic M2 binding densities in the hippocampus and temporal cortex of AD (cross-hatched bars) and control (solid bars) subjects as a function of *APOε4* allele copy number. Protocols for all cholinergic markers have been described (27). Data are means ± SEM from 2–27 brains. Statistically significant differences from normal control values are indicated: *, $P < 0.05$; **, $P < 0.001$.

poproteins in serum (37) and is markedly reduced in the brains of AD subjects (20) raises the possibility that synaptic integrity may be compromised as a consequence of impaired lipid homeostasis. Furthermore, triglycerides and fatty acids, which are precursor molecules in the biosynthesis of PC and PE (38), are also transported by apoE-containing lipoproteins in the serum (35). It is thus tempting to hypothesize that low levels of brain PC, PE, and cholesterol reported previously in AD subjects (18–21) could be caused by the low levels of apoE associated with the *APOε4* allele.

This working model is also consistent with that of Wurtman (39), who proposed that the unique propensity of cholinergic neurons to use choline for two purposes—namely, ACh and PC synthesis—may contribute to their selective vulnerability in AD. When physiologically active, cholinergic neurons may use free choline from the "reservoir" of membrane PC to synthesize ACh, hence altering their membrane and synaptic integrity (39). In that context, low levels of apoE in *APOε4* carriers and reduced transport of precursor molecules to cholinergic neurons could precipitate this cascade of events to the point of compromising choline metabolism and, indirectly, ACh synthesis. This model remains to be tested in apoE knockout mice,

which show impaired synaptic integrity (34) and marked cognitive decline (35).

This hypothesis and the effect of apoE4 on lipid homeostasis are both consistent with other membrane defects reported in AD subjects such as changes in membrane composition and fluidity in the brain and peripheral cells of AD patients (40–44).

The concept that synaptic plasticity and cholinergic integrity may be selectively compromised in apoE4 carriers was recently put forward by us (17) as both processes rely on intact brain lipid homeostasis and conceivably on apoE as well. Under these circumstances, drugs designed to take advantage of the residual cholinergic activity present in the AD brain should be more efficient in brains capable of plasticity and showing near normal ChAT and nicotinic receptor activities. This would be particularly appropriate for AChE inhibitor-based drug trials. To test this hypothesis, the apoE genotype was determined in 40 patients enrolled in a 30-week randomized controlled trial of a high-dose of tacrine (32), a potent, centrally active cholinesterase inhibitor (45).

Fig. 3 illustrates individual differences in ADAS scores as a function of apoE genotype. Overall, 83% of non-apoE4-
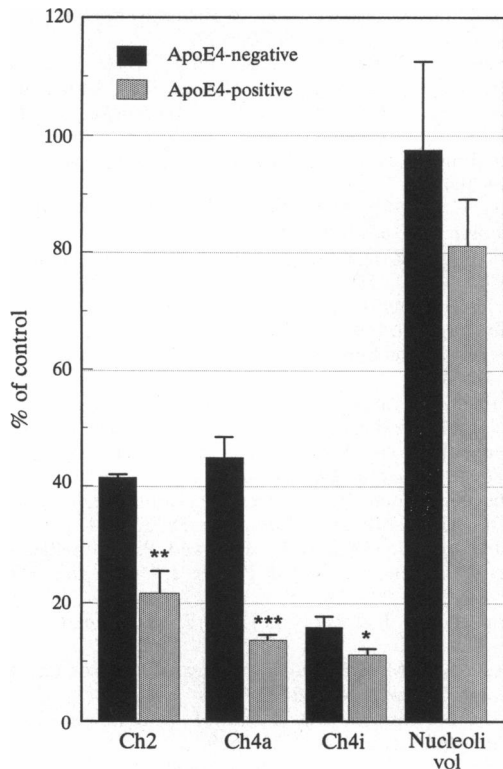
FIG. 2. Relationship between AChE-positive neuronal cell density (% of control) in Ch2 and Ch4 nucleus basalis of Meynert subdivisions and presence of the *APOε4* allele in AD subjects. Nucleolar volumes of surviving AChE-positive cells are shown in relation to the presence and absence of *APOε4* alleles. Bars represent means ± SEM for each group. Significant differences between AD groups (*n* = 4 for apoE4-negative subjects and *n* = 4 for apoE4-positive subjects) are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by *t* test.

Table 2. Analysis of change from baseline at 30 weeks in patients with AD from a tacrine study

Outcome measures	Non-E4 ($n = 18$) Mean	SD	E4 ($n = 22$) Mean	SD	P value
ADAS	−4.9	7.9	0.9	9.5	0.04*
ADAS-Cog	−3.9	5.8	0.3	6.9	0.04*
MMSE	1.9	3.3	1.2	4.2	0.56
IADL	0.9	2.7	0.9	3.0	0.92
PSMS	0.2	1.8	1.2	2.1	0.10
PDS	48.9	21.2	51.8	15.2	0.63
GDS	4.7	0.8	4.2	0.8	0.16
CIBIC	3.8	0.8	3.9	1.1	0.58

*Significant difference by Student's *t* test.

carrying patients had improvement in response to tacrine. In contrast, 60% of apoE4 patients were unchanged or worse after 30 weeks. These differences were analyzed by *t* test. Our results indicate that the patients not carrying apoE4 were significantly more likely to improve by 30 weeks (as measured by ADAS) than patients who were carriers of apoE4 (Table 2) ($P < 0.04$). A significant difference in favor of non-apoE4 patients being better responders was also seen in the ADAS-

Cog. This same pattern of improvement at 30 weeks was seen by MMSE, CIBIC, PSMS, PDS, GDS, and IADL scores, but the difference did achieve significance. The differences seen in ADAS-Cog scores with apoE genotype were analyzed with demographic variables for interaction. In exception to the general trend, four of the apoE4 patients were markedly improved from baseline. The patients were not different from the group as a whole with regard to age, sex, and education. Diffuse Lewy body disease is an entity recently suggested to be more responsive to tacrine therapy (46, 47). None of the apoE4 patients who improved had diffuse Lewy body disease.

The results are in good agreement with our initial working hypothesis (17). Subjects who do not carry the apoE4 allele should respond better to cholinergic drug therapies based on the functional use of residual cholinergic functions. Under these circumstances, apoE3 and/or apoE2 bearers showing sufficient residual ChAT activity should clearly benefit from an AChE-based therapy.

In summary, both pathological and clinical data clearly suggest that apoE4 genotype influences the function and integrity of the cholinergic system in the brain. Indeed, presynaptic cholinergic markers in *APOε3/ε3* AD subjects are relatively spared when compared to *APOε4/ε3* and *APOε4/ε4* AD carriers. Most importantly, this genetic susceptibility results in subgroups of AD patients, which respond differentially to cholinomimetic-based therapies like tacrine, with *APOε4* carriers being at a greater risk for loss of their ACh synthetic capacities and therefore less capable of responding. The presence of the *APOε4* allele could thus be one of the key factors responsible for individual variations in residual brain
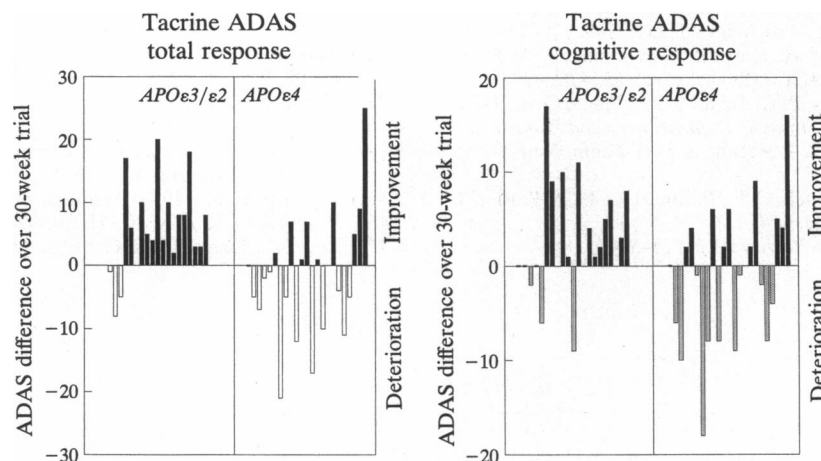

FIG. 3. Effect of *APOε4* allele copy numbers on tacrine drug responsiveness in AD subjects. Forty subjects enrolled in the 30-week randomized controlled trial of high-dose tacrine in patients with AD were selected from the original 663-subject cohort (32). Patients were selected prior to apoE phenotype determination and were blind to genotype. Phenotypic determination of the apoE genotype was performed as described (7) with frozen serum. Graph represents individual variation (ADAS score before and after drug trial) in total and cognitive performances as a function of *APOε4* allele incidence.

cholinergic innervation in AD and may be a useful predictor to clinical outcome of cholinergic/AChE inhibitor-based therapies. This observation alone should have a significant impact on the design of future cholinomimetic-based trials in AD in addition to focusing efforts to understand mechanisms involving apoE4 in the cholinergic deficit in AD.

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