Aggravated decrease in the activity of nucleus basalis neurons in 
Alzheimer's disease is apolipoprotein E-type dependent

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ABSTRACT As reported before, the metabolic activity of nucleus basalis neurons is reduced significantly in Alzheimer patients. Because the apolipoprotein E (ApoE) e4 genotype is a major risk factor for Alzheimer's disease (AD), we determined whether the decrease in metabolic activity in nucleus basalis neurons in AD is ApoE-type dependent. The size of the Golgi apparatus (GA) was determined as a measure of neuronal metabolic activity in 30 controls and 41 AD patients with a known ApoE genotype by using an image analysis system in the nucleus basalis of Meynert. A polyclonal antibody directed against MG-160, a sulfogalactoprotein of the GA, was used to visualize this organelle. There was a very strong reduction in the size of the GA in the nucleus basalis of AD patients. Furthermore, a strong and significant extra reduction in the size of the GA was found in the nucleus basalis neurons of AD patients with either one or two ApoE e4 alleles compared with Alzheimer patients without ApoE e4 alleles. Our data show that the decreased activity of nucleus basalis neurons in AD is ApoE e4 dependent and suggest that ApoE e4 participates in the pathogenesis of AD by decreasing neuronal metabolism.

Alzheimer's disease (AD) is the most common cause of dementia in elderly. This disorder is characterized by progressive memory loss, other cognitive impairments, and by neuropathological lesions, i.e., neuritic plaques, neurofibrillary tangles, and neurophil threads (1, 2). Epidemiological and molecular genetic studies have revealed that the genetic variation in apolipoprotein E (ApoE) is an important risk factor for AD (3–6).

Human ApoE is a 37-kDa protein encoded by a four-exon gene of 3.6 kb in length located on the long arm of chromosome 19. ApoE polymorphism consists of three types, i.e., ApoE e2, ApoE e3, and ApoE e4, which results in six different ApoE phenotypes in the population (6). ApoE e3, the most common isofrom, has a cysteine at residue 112 and an arginine at residue 158, whereas ApoE e4 has an arginine at both sites. ApoE e2 has a cysteine at both sites. ApoE e2, e3, and e4 have allele frequencies of ~0.08, 0.78, and 0.14, respectively (7, 8). The inheritance of one or two ApoE e4 alleles increases the risk of AD and decreases the age of onset of this disease (9), whereas ApoE e2 appears to reduce the risk of AD and increase the age of onset (10). The ApoE e4/4 genotype is associated with a mean age of AD onset of 60–70 yr in most populations studied. Few ApoE e4/4 individuals reach the age of 90 yr without developing AD (11–14). The presence of ApoE e4 has a direct impact on amyloid accumulation, neurofibrillary tangle formation, neurotrophin receptor loss (15), and cholinergic deficits (15–18).

The suggestion that reduced neuronal activity in AD brains may by itself be a crucial hallmark for AD (19, 20) raised questions on the nature of the relationship between AD pathology and neuronal activity. In a series of studies, we established that plaques, tangles, and decreased neuronal activity as determined by the size of the Golgi apparatus (GA) occur independently from each other in various brain areas of AD patients (20–23). The nucleus basalis of Meynert (NBM) is neuropathologically severely affected in AD and also shows severely decreased neuronal activity (20). As a measure of neuronal metabolic activity that can be applied to formalin-fixed paraffin-embedded postmortem material, we used the size of the GA. It has been shown that all newly synthesized proteins destined for fast axonal transport are processed through the GA (24) and that the GA is involved in many physiological posttranslational modifications including the transport and targeting of a variety of proteins destined for secretion, the plasma membrane, and lysosomes (25, 26). Therefore, the decreased size of the neuronal GA reflects an impairment of protein processing. Because ApoE e4 is one of the major risk factors for AD, in the present study we examined whether there is a relationship between the reduction of the size of the GA and the size of NBM neurons and the type of ApoE in AD patients. In each Alzheimer patient the ApoE genotype was determined, the GA of the NBM neurons visualized by immunocytochemistry, and the size of the organelle measured by image analysis.

Indeed, a clearly reduced neuronal activity of NBM neurons was found in AD brains compared with that of nondemented controls. Moreover, a similar extra decreased neuronal activity of NBM neurons was found in the presence of ApoE e4 alleles in AD, which suggests that ApoE e4 alleles participate in the pathogenesis of AD by decreasing neuronal metabolic rate.

MATERIALS AND METHODS

Tissue Collection. Brains from 30 nondemented controls ranging in age from 29 to 94 (58.4 ± 1; mean ± SEM) and 41 Alzheimer patients ranging in age from 40 to 98 yr (72.8 ± 2) were obtained at autopsy (see Table 1 for clinico-pathological information). One AD patient was ApoE e2/3 (40-yr-old), 15 were of the ApoE e3/3 genotype (69.9 ± 3.1 yr), 16 of the ApoE e3/4 genotype (75.3 ± 3.6 yr), and 9 of the genotype e4/4 (73.2 ± 3.1 yr). AD was diagnosed clinically based on National Institute of Neurological and Communication Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria, and the diagnosis "probable AD" was established by excluding other causes of dementia (27). The patients had a global deterioration scale of 4–7 for severity of dementia (ref. 28; Table 1). The patients suffered between 3 and 14 yr of dementia before death (Table 1). The clinical diagnosis AD was neuropathologically confirmed by systematic study of the following structures: the temporal superior gyrus, orbital gyrus, cingulate gyrus, hippocampus, striatum, and

Abbreviations: AD, Alzheimer's disease; ApoE, apolipoprotein E; GA, Golgi apparatus; NBM, nucleus basalis of Meynert.

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Table 1. Cing-lopathological information on controls and Alzheimer patients with different ApoE genotypes

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Bw, g</th>
<th>PMD, hr</th>
<th>Fix, d</th>
<th>GDS</th>
<th>ApoE Genotype</th>
<th>Disease duration, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Control</td>
<td>58.4 ± 3.5</td>
<td>1,265 ± 30</td>
<td>17.42 ± 3.35</td>
<td>49 ± 15</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1</td>
<td>Alzheimer</td>
<td>40</td>
<td>1,410</td>
<td>2.83</td>
<td>28</td>
<td>7</td>
<td>2/3</td>
<td>60</td>
</tr>
<tr>
<td>15</td>
<td>Alzheimer</td>
<td>69.9 ± 3.1</td>
<td>1,071.3 ± 38.87</td>
<td>4.01 ± 0.24</td>
<td>32.5 ± 2.8</td>
<td>6.2 ± 0.26</td>
<td>3/3</td>
<td>90.0 ± 12</td>
</tr>
<tr>
<td>16</td>
<td>Alzheimer</td>
<td>75.3 ± 3.6</td>
<td>1,168.7 ± 52.72</td>
<td>4.13 ± 0.20</td>
<td>31.0 ± 2.8</td>
<td>6.5 ± 0.12</td>
<td>3/4</td>
<td>96.0 ± 8</td>
</tr>
<tr>
<td>9</td>
<td>Alzheimer</td>
<td>73.2 ± 3.1</td>
<td>1,180.2 ± 48.64</td>
<td>4.55 ± 0.38</td>
<td>29.7 ± 2.1</td>
<td>6.33 ± 0.44</td>
<td>4/4</td>
<td>105.3 ± 10</td>
</tr>
</tbody>
</table>

No, Number of patients; PMD, postmortem delay (in hours); fix, fixation time (in days); BW, brain weight (in grams); GDS, Global Deterioration Scale (28).

Immunocytochemistry. A polyclonal antibody was raised against immunoaffinity purified MG-160, a sialoglycoprotein of the medial cisternae of the rat neuronal GA. The specificity of this antibody for GA membranes had been established previously by light and ultrastructural immunocytochemistry (29–31). For the optimal retrieval of the MG-160 antigen, we used microwave oven heating before incubation with the primary antibody (32, 33).

For an extensive description of the immunocytochemical procedures, see Salehi et al. (20). In brief, the sections were incubated with anti-MG-160 (first antibody) and diluted 1:800 for 1 hr at room temperature followed by an overnight incubation at 4°C. Subsequently, the sections were washed in tris-buffered saline and incubated with biotinylated goat-anti-rabbit Ig (second antibody; Vector Laboratories) at a dilution of 1:500 for 1 hr at room temperature and finally with avidin-biotin-horseradish peroxidase (Vector Laboratories) at a dilution of 1:1500 for 1 hr at room temperature. 3,3'-Diaminobenzidine-tetrahydrochloride (Sigma) was used as chromogen. Staining enhancement was obtained by adding ammonium nickel sulfate (2.2 mg/ml). The sections were then dehydrated in graded ethanol and xylene and coverslipped with Entellan (Merck).

Nucleus Basalis. The NBM, or Ch4 area according to Mesulam's nomenclature (34), provides the major cholinergic innervation for the cerebral cortex and the amygdala (34, 35). In the human brain, in addition to choline acetyltransferase, Ch4 neurons also express acetylcholinesterase, calbindin-D28k, the tyrosine kinase receptors A, B, and C, and the low affinity neurotrophin receptors (36–38). The NBM is severely affected in AD. Originally it was presumed that massive neuronal death is one of the major hallmarks of AD, in the NBM and other regions (39–41). However, recent studies have indicated that neuronal atrophy rather than cell loss is the main phenomenon in the NBM in AD (20, 42, 43).

Morphometry. Because the NBM is a very extensive cell system, the measurements were performed in a standardized part of the NBM, i.e., in the medial subdivision of Ch4 (34). NBM-containing sections were selected on the basis of a standardized location of the fornix, anterior commissure, optic tract, and supraoptic nucleus (20).

Measurement of the GA surface was performed by using an IBAS image analysis system [Kontron AT-based system]. The image analysis system was connected to a Bosch TY9KB TV camera equipped with a chalycon tube mounted on a Zeiss microscope. The microscope was equipped with planapo- objectives. All measurements were performed by using a 560-nm filter, which coincides with the maximum absorption of the diaminobenzidine/nickel sulfate precipitate in the sections. For an extensive description of the analysis procedures, see Salehi et al. (20).

**Statistical Methods.** The differences in the mean values of the neuronal GA and mean cellular profile area among AD groups with different ApoE genotypes were tested by using the Kruskal–Wallis test and Mann–Whitney U test. To test the correlation between different parameters such as fixation time, postmortem delay, and age of the subjects and the mean size of the GA, the Pearson’s correlation coefficient was used. A P-value <0.05 was considered to be significant.

**APOE Genotyping.** APOE genotyping was performed on frozen tissue from the cerebellum of the Alzheimer patients. The genotype of each extracted DNA sample was determined by PCR amplification by using the primers 5'-ATAATAT-AAAAATATTAAATACAGAATTCCCGCCGTCCTGGTCACAC-3' and 5'-TAAGCTTGGCCACGCTGTCCAAAGGAC-3'. Then the PCR product was digested by CfoI, and fragments were separated by electrophoresis in a 5% agarose gel (44).

**RESULTS AND DISCUSSION**

The immunocytochemical visualization of the GA in the NBM neurons revealed a cytoplasmatic staining with a perinuclear distribution in both neurons and glia, consistent with the location of the GA (Fig. 1A–B). Qualitative microscopic analysis showed that the area occupied by the GA in the cytoplasm of NBM neurons in control subjects was clearly larger than that of Alzheimer patients. Furthermore, the GA size in Alzheimer patients with ApoE genotype e3/3 (Fig. 1A) was generally larger than that of Alzheimer patients with ApoE genotype e3/4 (Fig. 1B) or e4/4. In addition to this reduction in GA size in Alzheimer patients with ApoE genotype e3/4 or e4/4, a reduction in the cell profile area was observed in these patients as compared with Alzheimer patients with ApoE genotype e3/3.

Image analysis confirmed the qualitative microscopic impression. The mean area of the GA in control subjects (127.8 ± 10.4 μm²; mean GA size ± SEM) was significantly (P < 0.001) larger than that of Alzheimer patients (63.7 ± 4.3 μm²). As far as the Alzheimer group was concerned, the Kruskal–Wallis test showed significant (P = 0.002) differences in GA size and mean profile area among the three groups of patients with different ApoE genotypes (Fig. 2). Alzheimer patients with ApoE genotype e3/3 had a mean GA size of 85.1 ± 5.2 μm², whereas Alzheimer patients with ApoE genotype e3/4 had a mean GA size of 51.9 ± 5.9 μm². The patients with ApoE genotype e4/4 had a mean GA size of 48.8 ± 8.1 μm². The mean GA size of Alzheimer patients with ApoE genotype e3/4 or e4/4 was significantly (P < 0.001) lower than the mean GA size of Alzheimer patients with ApoE genotype ApoE e3/3 (Fig. 2). The size of the GA of Alzheimer patients with ApoE genotype e4/4 was not different (P = 0.760) from that of Alzheimer patients with ApoE genotype e3/4. The cell profile area of Alzheimer patients with ApoE genotype e3/4 (254.1 ± 17.0 μm²) or 4/4 (187.3 ± 23.0 μm²) was significantly lower (P < 0.001) than that of Alzheimer patients with ApoE genotype e3/3 (346.1 ± 12.1 μm²).
Fig. 1. Immunocytochemical staining of the Golgi apparatus in Alzheimer patients with ApoE genotype e3/3 and ApoE genotype e3/4. Note the reduction in Golgi size in the nucleus basalis neurons of Alzheimer patients with ApoE genotype e3/4 (B) vs. the Alzheimer patients with ApoE genotype e3/3 (A). (Bar = 30 μm.)

Using the Pearson’s test, no significant correlation could be found between fixation time postmortem delay, disease duration, and age of the subjects and the mean GA size either in controls or in Alzheimer patients with different ApoE genotypes. Moreover, no significant correlation could be found between fixation time or postmortem delay of the subjects and the cell profile area of the either controls or Alzheimer patients with different ApoE genotypes. Nor was the cell profile area dependent on the age of the subjects (Table 2). Furthermore, there were no significant differences in age (P = 0.296), brain weight (P = 0.249), postmortem delay (P = 0.507), fixation time (P = 0.872), global deterioration scale (P = 0.652), or duration of the disease (P = 0.451) among the three groups of AD patients (Table 1) with either one or two or without any ApoE e4 alleles.

Mutations in different genes, including amyloid precursor protein, presenilin-1 and presenilin-2, are invariably associated with AD (45–48). However, the ApoE e4 variant associated with AD also can be found in quite elderly, cognitively normal individuals (49), indicating that ApoE e4 should be considered as a risk factor for AD. Furthermore, polymorphism in specific regions of the ApoE gene might be of importance for the occurrence of AD (50).

![Graph showing the size of the mean Golgi apparatus in controls and Alzheimer patients with ApoE genotype e3/3 compared with Alzheimer patients with ApoE genotype e3/4 and e4/4. Note the clear reduction in the size of Golgi apparatus in Alzheimer patients with one or two ApoE e4 alleles, compared with Alzheimer patients without ApoE e4 alleles. There is no significant difference (P = 0.760) in Golgi apparatus size between Alzheimer patients with one ApoE e4 allele and two e4 alleles.]

The mechanism of involvement of ApoE in AD is not known. It was hypothesized (i) that ApoE affects the deposition of amyloid by changing the clearance, the deposition or the generation of β-amyloid, or (ii) interacts with the microtubule-associated protein τ, in that ApoE e4 does not protect τ from aggregation into neurofibrillary tangles. In addition, a role of ApoE in neuronal repair and degeneration has been suggested (51–53), and it was proposed that ApoE e4 might be less able to support neuronal survival during neurodegeneration than ApoE e2 or e3. The prevalence of ApoE indeed correlated with β-amyloid accumulation (3, 5, 55) and neurofibrillary tangle formation (55, 56), supporting the possibility of a causal relationship between ApoE and the development of AD neuropathology. Moreover, the role of ApoE in the cascade of cholesterol and phospholipid transport and re-uptake and the involvement of these mechanisms in repair in brain aging and AD (57) has been taken into consideration. It was proposed that cholesterol, released during the breakdown of the synaptic terminal, is transported by ApoE to neurons undergoing reinnervation (57). Because the transport of cholesterol and other lipoproteins play a central role in synaptogenesis, it is possible that Alzheimer patients differing in their ApoE phenotype also differ in their capacities of synaptogenesis. Recent in vitro data have shown that in the presence of a lipid source, ApoE e3 enhances and ApoE e4 inhibits neurite outgrowth in neuronal cell cultures (58). The results of the present study suggest a new mechanism for the involvement of ApoE e4 because it was found that the neurons of Alzheimer patients with ApoE genotype e3/4 or e4/4 are less metabolically active than those of Alzheimer patients with ApoE genotype ApoE e3/3. Interestingly, we could not find a significant difference in the size of the GA between Alzheimer patients with ApoE genotype e4/4 and those with e3/4. This result indicates that the relationship between the size of the GA and the number of ApoE e4 alleles is not dose-dependent.

<table>
<thead>
<tr>
<th>P-value of correlation* between the GA size, cell profile area and clinico-pathological parameters of the subjects used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td>Alzheimer</td>
</tr>
<tr>
<td>Alzheimer</td>
</tr>
</tbody>
</table>

PMD, postmortem delay; *, Pearson’s correlation test; Fix, fixation time; GDS, Global Deterioration Scale (28).
and that there may be a threshold effect in the NBM, as was found for the cholineretic deficit in the NBM (18).

We have shown in the present study that the presence of one or two ApoE e4 alleles is accompanied by a decreased metabolic activity of neurons as indicated by the diminished size of the GA, which was interpreted in our studies as an indicator of reduced neuronal metabolic activity. However, it may well be that the GA itself is a direct target of ApoE e4 alleles. In support of this possibility, Verde et al. (59) have shown that ATP depletion blocks the export of proteins from the endoplasmic reticulum to the intermediate compartment of the Golgi complex, which may lead to GA shrinkage (59).

Our present study clearly confirms our previous work (20) that in AD patients the GA (63.70 ± 4.3) is approximately one-half the size of that in controls (127.83 ± 10.4). One of the questions that we are currently trying to address is whether the number of ApoE e4 alleles in controls is related also to the GA size. To do so, we are optimizing a method to determine the number of ApoE e4 alleles in the paraffin-embedded tissue of the controls. However, an extremely large collection would be needed to obtain a group of neuropathologically confirmed control cases with ApoE e4 alleles. No case with ApoE e4/4 genotype was found in the collection of the Netherlands Brain Bank out of 117 nondemented controls.

One of the most consistent biochemical abnormalities in AD brains is a cholinergic deficit, as evidenced by a loss of choline acetyltransferase and acetycholinesterase in the neocortex and hippocampus, and a degeneration of cholinergic neurons in the NBM (20, 39). Recent studies have indicated that Alzheimer patients carrying an ApoE e4 allele have a more severe cholinergic deficit in the hippocampus, temporal cortex (18), and frontotemporal cortex (15). The reduced activity of choline acetyltransferase in the temporal cortex reported by Poirier et al. (18) in Alzheimer patients was found to be similar in patients with either one or two ApoE e4 alleles, just as we found a similar reduction in metabolic rate in the NBM of these two patient groups. All these data support the notion that ApoE may play a crucial role in the metabolic activity of the cholinergic neurons of the NBM. Our data suggest that ApoE e4 may be a risky factor for the development of AD by reducing neuronal metabolism. It may be presumed that a lower protein synthetic ability of the NBM neurons in AD predisposes them to neurodegeneration (19).

An enhanced dose-dependent burden of β-amyloid has been observed in ApoE e4 carriers (55, 56, 60). It has been shown that inhibition of energy metabolism can influence amyloid precursor protein processing, leading to decreased secretion of nonamyloidogenic fragments of amyloid precursor protein (61–63). One may suggest, therefore, that ApoE e4 alleles increase the burden of amyloid by inhibition of the energy metabolism. On the other hand, we found no clear relationship between the GA size and the distance of neurons from neuritic plaques (A.S., C.W. Pool, M.M., R. Ravid, N.K. Gonatas, and D.F.S., unpublished data), pleading against such a local inhibitory effect of amyloid on neuronal metabolism.

The proposal that ApoE influences the metabolic activity of neurons may have clinical implications because it supports the idea that in cholinergic treatment of Alzheimer patients the ApoE genotype should be taken into account. The differential responses to drugs with cholinergic enhancing effects, such as tacrine of AD individuals with different ApoE types could be explained by our data. This possibility is supported by the study of Poirier et al. (18), who showed that patients without e4 alleles responded significantly better than AD patients carrying the ApoE e4 alleles to a 30-wk treatment with tacrine.

The next question in our study will be what the mechanism is by which ApoE subtypes may influence the rate of metabolism.