

## NO EVIDENCE FOR CHOLINERGIC PROBLEMS IN APOLIPOPROTEIN E KNOCKOUT AND APOLIPOPROTEIN E4 TRANSGENIC MICE

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**Abstract**—The  $\epsilon 4$  allele of the apolipoprotein E gene constitutes the major genetic risk factor to develop Alzheimer's disease. If and how this protein contributes to the pathological cascade of Alzheimer's disease is not known. The  $\epsilon 4$  allele particularly affects the cholinergic defect, which is one of the most consistent neurotransmitter problems in an Alzheimer's disease brain.

We have analysed several parameters of the cholinergic system in brain of apolipoprotein E knockout mice as well as in transgenic mice overexpressing human apolipoprotein E4. We analysed the distribution of cholinergic fibers, the number and morphology of cholinergic neurons and the enzymatic activity of acetylcholinesterase and choline acetyltransferase in different brain regions. Finally, we analysed the distribution and the binding parameters of [ $^3$ H]hemicholinium-3, a specific marker for the high affinity choline transporter in different brain sections and regions.

This extensive effort failed to show any consistent difference in the cholinergic parameters studied, in either the apolipoprotein E4 transgenic mice or in the apolipoprotein E knockout mice, compared to age-matched non-transgenic mice. We conclude that the apolipoprotein E4 is not deleterious *per se* for the cholinergic system in mouse brain. © 2000 IBRO. Published by Elsevier Science Ltd.

**Key words:** apolipoprotein E, Alzheimer's disease, cholinergic system, transgenic mice.

Alzheimer's disease (AD) is the most common type of dementia, manifesting as a severe deterioration of different mental functions.<sup>14</sup> Familial AD is due to mutations in the amyloid precursor protein or in the presenilin genes.<sup>37</sup> In sporadic AD, the frequency of the  $\epsilon 4$  allele of the apolipoprotein E (ApoE) gene is increased and constitutes the major genetic risk factor (40–50% of AD patients), decreasing the age of onset.<sup>13,49,31</sup>

The ApoE protein is an important determinant of lipid transport in non-nervous tissues (for review see Ref. 26). In the central nervous system (CNS) it is the principal lipoprotein of cerebrospinal fluid. The ApoE mRNA is mainly present in astrocytes, but also in neurons.<sup>11,58</sup> The lipoproteins contain principally cholesterol ester, cholesterol and phospholipids.<sup>39,44</sup> The ApoE protein plays an important role in lipid metabolism by mediating the binding of lipoproteins to the low-density lipoprotein receptor and the low-density lipoprotein-receptor-related protein.<sup>39,29</sup> In response to damage, astrocytes in the CNS synthesize and release ApoE within the lesion, presumably to scavenge for cholesterol and phospholipids originating from cellular and myelin debris, while others suggested a role for ApoE in synaptic plasticity.<sup>41</sup>

Post-mortem AD brain is characterized by the presence of

amyloid plaques, neurofibrillary tangles, synaptic loss, cell loss and neurotransmitter derangement.<sup>53,23,21</sup> A consistent finding in AD is the cholinergic deficit, although other neurotransmitter systems are impaired.<sup>20,42</sup> The cholinergic problem continues to attract interest because its magnitude parallels the severity of dementia.<sup>38,8</sup> Drugs that target the cholinergic system are at this moment the main type available for symptomatic treatment of AD patients.<sup>36,18</sup>

The presence of  $\epsilon 4$  alleles appears to have a direct impact on amyloid accumulation, neurofibrillary tangle formation, neurotrophin receptor loss and on the severity of the cholinergic deficit.<sup>51,35,52,40,3,6,47</sup> The presence of ApoE4 reduced the metabolism of cholinergic neurons and altered the plasticity of cholinergic dendrites in AD patients.<sup>7,47</sup> In addition, AD patients carrying  $\epsilon 4$  alleles respond less well to cholinergic therapy.<sup>40</sup> Reduced levels of cholinergic markers have also been reported in normal subjects carrying  $\epsilon 4$  alleles.<sup>3</sup> The combined evidence underlines the possibility that the ApoE4 isoform may have a deleterious effect on the cholinergic circuitry. On the other hand, a cholinergic deficit has also been claimed for ApoE knockout mice,<sup>22,12</sup> although controversy remains at this point.<sup>4,16</sup> One hypothesis proposed that cholinergic neurons could be more vulnerable or dependent on lipid metabolism mediated by ApoE because they use phosphatidylcholine as the source of choline for acetylcholine synthesis.<sup>57</sup>

The controversy prompted us to analyse whether ApoE knockout mice and transgenic mice overexpressing the human ApoE4 isoform in their brain develop cholinergic defects.

Different aspects of the cholinergic system are affected in AD and possibly influenced by the presence of the  $\epsilon 4$  allele of ApoE.<sup>32,56,9,20,5</sup> Among these we studied: (i) the extent of

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**Abbreviations:** AChE, acetylcholinesterase; AD, Alzheimer's disease; ApoE, apolipoprotein E; ApoE o/o, ApoE knockout; ChAT, choline acetyltransferase; CNS, central nervous system; DB, diagonal band; EDTA, ethylenediaminetetra-acetate; HACHT, high affinity choline transporter; iso-OMPA, tetraisopropylpyro-phosphoramidate; MS, medial septum; NBM, nucleus basalis magnocellularis; TBS, Tris-buffered saline.

cholinergic fiber innervation in the cortex and hippocampus by acetylcholinesterase (AChE) histochemistry; (ii) the AChE and choline acetyltransferase (ChAT) activity levels in different areas of the brain; (iii) the cholinergic cell number and gross morphology by immunohistochemistry against ChAT; and finally we included (iv) the study of high affinity choline transporter (HAcHT), a well known presynaptic marker.

This effort failed, however, to demonstrate a consistent cholinergic problem in the brain of ApoE4 transgenic mice or in the ApoE knockout mice. The results demonstrated that the ApoE4 isoform is not deleterious *per se* for the cholinergic system, and that reported claims of a cholinergic deficit in ApoE knockout mice must be due to factor other than the mere absence of ApoE.

## EXPERIMENTAL PROCEDURES

### Materials

The rat monoclonal antibody against ChAT was purchased from Boehringer (Mannheim, Germany). Secondary antibodies conjugated with biotin were from DAKO (Glostrup, Denmark). [<sup>3</sup>H]Acetyl coenzyme A and [<sup>3</sup>H]hemicholinium-3 were purchased from NEN<sup>th</sup>, Life Science Products (Boston, MA, U.S.A.). Atropine, hemicholinium-3, tetraisopropylpyro-phosphoramidate (iso-OMPA), 1,5-bis (4-allyldimethylammoniumphenyl) pentan-3-one dibromide (Bw284c51), choline iodide, acetyl coenzyme A, hemicholinium-3, acetylthiocholine iodide, tetraphenylborate, and 3-heptanone were purchased from Sigma (St. Louis, MO, U.S.A.).

### Mice

Transgenic mice overexpressing the human ApoE4 protein in the brain were generated as described<sup>33,34</sup> and maintained in the FVB genetic background. Their phenotype characterization is reported elsewhere.<sup>54</sup> Mice deficient in ApoE (ApoE o/o) were generated<sup>55</sup> and maintained in the C57Bl/6 background. All mice were identically housed in a temperature and humidity controlled vivarium, kept on a 12-h dark/light cycle (light on 07:00 EST), with free access to food and water. Age-matched non-transgenic mice of FVB strain were used as control for ApoE4 transgenic mice (five to six months) and age-matched non-transgenic mice of C57Bl/6 strain for ApoE o/o (six to nine months). Experiments were carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to reduce the number of animals used and to minimize animal suffering.

### Immunohistochemistry

Mouse brain was fixed by cardiac perfusion under deep anesthesia with 5 ml saline followed by 20 ml cold fixative (4% paraformaldehyde, 0.2% picric acid, 0.1 M phosphate buffer, pH 7.4) and post-fixed for 2 h in the same solution. Fixed brain was stored at 4°C, in 30% sucrose, 0.1 M phosphate buffer (pH 7.4). Free-floating coronal serial sections (40 µm) were collected in 0.1 M phosphate buffer (pH 7.4) and processed as follows: (i) incubation for 1 h at room temperature with 10% rabbit serum, 0.1 M Tris, 150 mM NaCl, pH 7.4 (TBS); (ii) overnight incubation at 4°C with antibody to ChAT (diluted 1:200 in 0.2% Triton-X-100, 20% rabbit serum, 0.2% albumin in TBS); (iii) incubation for 30 min at room temperature with biotin-conjugated rabbit anti-rat IgG (diluted 1:300 in 20% non-immune rabbit serum, 0.1% albumin in TBS); and (iv) incubation with avidin conjugated to peroxidase (ABC, DAKO, Denmark), followed by visualization of peroxidase activity with diaminobenzidine (1 mg/ml) and H<sub>2</sub>O<sub>2</sub> (0.01%) in TBS.

### Acetylcholinesterase enzyme histochemistry

Mouse brain was fixed by cardiac perfusion under deep anesthesia with 5 ml of saline followed by 20 ml of cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brain was post-fixed overnight in the same fixative and placed in 30% sucrose, 0.1 M phosphate buffer (pH 7.4) for two days at 4°C. Serial coronal frozen sections (40 µm)

were collected in 0.1 M phosphate buffer (pH 7.4) and were incubated as free-floating sections, as described.<sup>27</sup>

### Morphometric analysis of cholinergic cells

Basal forebrain cholinergic neurons were defined from anatomical landmarks, taken from the mouse brain atlas.<sup>19</sup> The anterior commissure, anterior and the lateral ventricles defined the ventral border of the medial septum (MS). The meeting of the body of the corpus callosum at the midline marked the anterior boundary and the midline crossing of the anterior commissure and the appearance of the fornix marked the posterior boundary. Five thick coronal sections (40 µm) were analysed to cover the complete medial septum, with 120 µm intervals between each, to avoid counting the same cell twice (coordinates from Bregma: 1.10 mm to 0.5 mm). The cholinergic cells of the horizontal limb of the diagonal band (DB) were defined as the group of immunopositive cells residing below the anterior commissure, anterior and the anterior commissure, posterior. The appearance of the anterior commissure, posterior marked the anterior boundary and the connection of the lateral ventricles marked the posterior boundary. Seven thick coronal sections (40 µm) were analysed with 80-µm intervals (coordinates from Bregma: 0.38 mm to -0.34 mm). The cholinergic cells of the nucleus basalis magnocellularis (NBM) straddle the border between the lateral globus pallidus and the internal capsule. The anterior boundary was defined as the connection of lateral ventricles (coordinate from Bregma: -0.34 mm) and the posterior boundary was defined with the dorsal hippocampus (coordinates from Bregma: -1.34). Seven thick coronal (40 µm) sections were analysed with 80-µm intervals. In each mouse brain the same number of sections and similar total distance for each nucleus were analysed for cholinergic cell quantification to guarantee that similar volumes of each nucleus were quantified.

For each section, immunopositive neuronal profiles were counted manually on photographs taken with a ×10 objective on a Zeiss IM35 inverted optic microscope. The final magnification of the photograph was four times the negative (10×15 cm). This procedure allows clear visualization of cholinergic cells. The total area covered for each photograph was 670×1000 µm<sup>2</sup>, enough to include all immunopositive cells of each cholinergic nucleus analysed.

The number of neuronal profiles counted in each set of five or seven sections yielded the total number of cells per animal, corrected according to Abercrombie.<sup>1</sup>

In addition, ChAT immunopositive cells were counted on images digitized on a Leica DMR microscope (×20 objective), equipped with a CCD video camera and connected with a computerized image analysis system (AIS/C, Imaging Research, Canada). The image was incremented by 200% and, using a viewing box, neurons were identified on the screen and counted. Two fields of 665×470 µm<sup>2</sup> were considered for the analysis of the MS, one for the DB and two for the NBM. Both procedures gave very comparable numbers of neuronal profiles.

### Size of cholinergic neurons

A total of 100 neurons was assessed in each nucleus in each animal by measurement of cross-sectional area (a total of 400 neuronal profiles per genotype). Using a objective ×20, fields of 665×470 µm were digitized and incremented by 400%. The image was processed with the detail extractor function that enhances the contrast and accentuates the edges of the neuronal profiles. The neuronal profiles were identified on screen and the cross-sectional area (µm<sup>2</sup>) was calculated. The results are presented as the mean cross-sectional area (neuronal size) ± S.E.M.

### Biochemical assays

ApoE4 transgenic mice, ApoE o/o mice and age-matched non-transgenic mice were killed by cervical dislocation and brain was removed. The hippocampus and the cortex were carefully dissected, washed in 0.1 M phosphate-buffered saline and 150 mM NaCl (pH 7.4) and weighed. The tissue was homogenized in nine volumes of ice cold buffer A [50 mM Tris-HCl, pH 7.4, 1.0 M NaCl, 2.5 mM EDTA and 50 mM MgCl<sub>2</sub> (pH 7.4)] containing a cocktail of proteinase inhibitors and using a glass-teflon homogenizer. The suspension was centrifuged at 150,000g at 4°C for 1 h in a Beckman TLA 100.4 rotor to recover the salt-soluble (SS) fraction. The pellet was resuspended in buffer A containing 1% Triton X-100, and centrifuged at 30,000g at 4°C for 1 h to recover the Triton X-100-soluble (TS) fraction. This method allows the extraction of more than 90% of the total AChE enzymatic

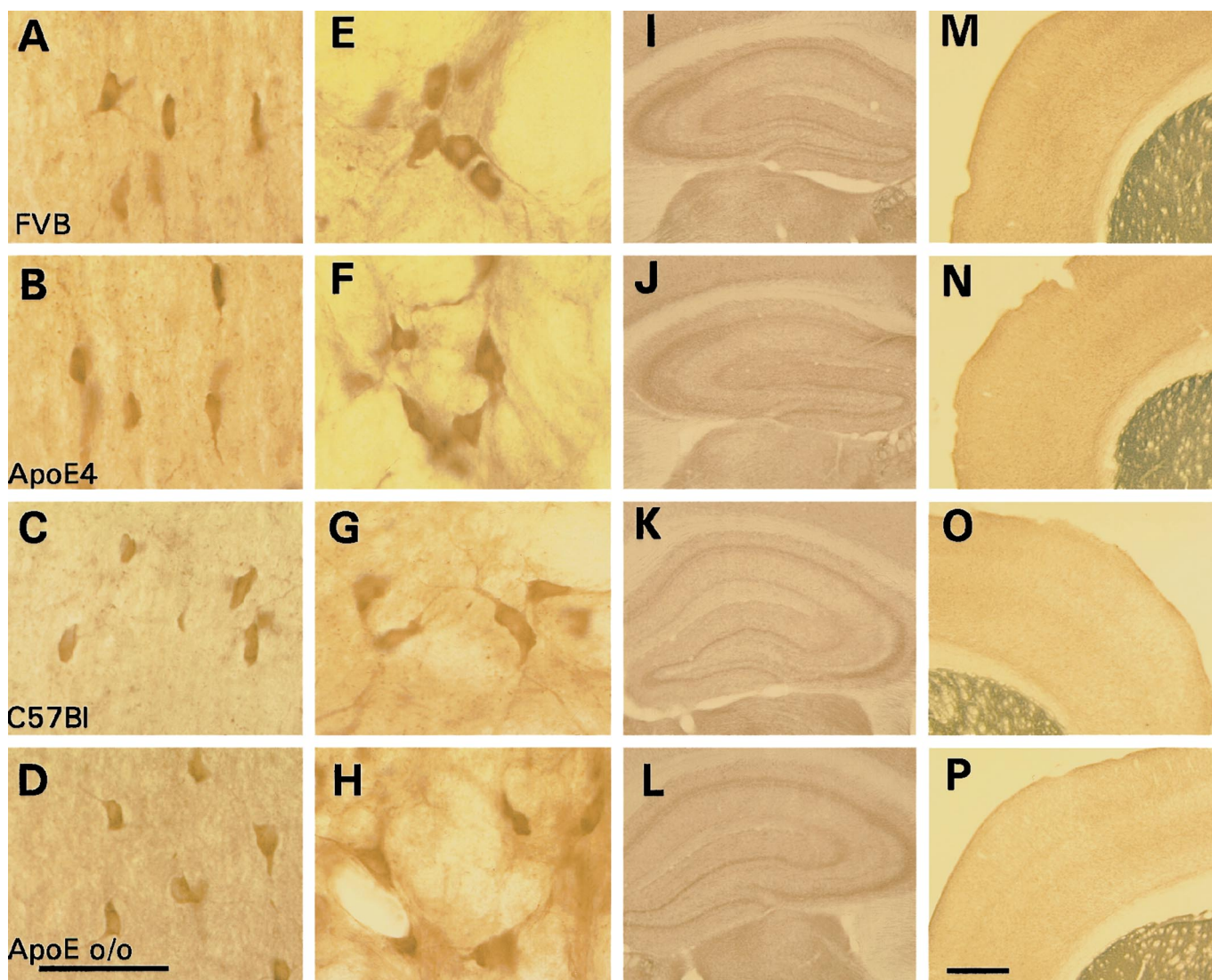


Fig. 1. Immunohistochemistry for choline acetyltransferase in the brain of non-transgenic, ApoE *o/o* and ApoE4 transgenic mice. ChAT-immunoreactive neurons in the medial septum (A–D) and in the nucleus basalis magnocellularis (E–H). Scale bar = 50  $\mu$ m. View of the hippocampus (I–L) and frontal cortex (M–P) following AChE histochemistry. Scale bar = 500  $\mu$ m. A, E, I, M, non transgenic mice from the FVB strain. B, F, J, N, ApoE4 transgenic mice. C, G, K, O, non-transgenic mice from the C57Bl strain. D, H, L, P, ApoE *o/o* mice.  $n = 4$ .

activity.<sup>50</sup> The protein concentration was measured by the method of Bradford.<sup>10</sup>

#### Acetylcholinesterase enzymatic activity assay

AChE activity was determined by the method of Ellman.<sup>15</sup> Briefly, 5  $\mu$ l brain extract was mixed with 200  $\mu$ l of reaction buffer (0.5 mM phosphate buffer, pH 7.4, 0.02% DTNB, 0.02% acetylthiocholine and 0.1 mM iso-OMPA), and after 5 min incubation at room temperature, the reaction was stopped with 1  $\mu$ M Bw284c51, a potent AChE inhibitor. The optical density was measured at 405 nm (Victor multilabel counter, Wallac). AChE activity was expressed as relative units and normalized to the protein content of the samples.

#### Choline acetyltransferase enzymatic activity assay

ChAT activity was measured as described.<sup>17</sup> Briefly, 5  $\mu$ l of brain extract homogenate was added to 10  $\mu$ l reaction buffer containing 75 mM sodium phosphate, pH 7.4, 600 mM NaCl, 40 mM MgCl<sub>2</sub>, 0.1 mM Bw284c51, 0.05% bovine serum albumin, 10 mM choline iodide, and 0.3 mM [<sup>3</sup>H]acetyl-CoA (specific activity of 40 mCi/mmol). The mixture was incubated for 30 min at 37°C and the reaction was stopped by the addition of 150  $\mu$ l of tetraphenylboron (75 mg/ml in 3-heptanone), gently mixed and centrifuged. One hundred microliters of the organic top layer were removed and placed into scintillation

vials and counted. After correction for background (no brain extract) ChAT activity was calculated (normalized to the protein content of the samples) and expressed relative to the levels in brain of non-transgenic mice.

#### High affinity choline transporter assay

The high affinity choline transporter (HACHT) was assayed with [<sup>3</sup>H]hemicholinium-3 as described.<sup>24</sup> Serial frozen coronal brain sections (16  $\mu$ m), from the level of the genu of the corpus callosum to the ventral hippocampus, were mounted on glass-slides coated with poly-lysine to reduce non-specific binding of [<sup>3</sup>H]hemicholinium-3. The sections were air-dried and kept at -70°C until used. Sections were equilibrated at room temperature and incubated for 30 min in 50 mM glycine-glycine (pH 7.8), 200 mM NaCl and six different concentrations, ranging from 1.25 to 30 nM [<sup>3</sup>H]hemicholinium-3 (120 Ci/mmol). After washing twice for 2 min in cold buffer, followed by rinsing in cold water, the sections were air-dried and exposed to [<sup>3</sup>H]Hyperfilm (Amersham, U.K.) for two weeks. Non-specific binding was determined by addition of 10  $\mu$ M hemicholinium-3 to the incubation mixture. Plastic embedded tritium standards ([<sup>3</sup>H]Micro-scale, activity levels, 3–110 nCi/mg, Amersham, U.K.) were co-exposed with tissue section. Autoradiographic images from the sections were scanned and the optical density from the hippocampus, frontal cortex and entorhinal cortex was quantified by using the analytical imaging

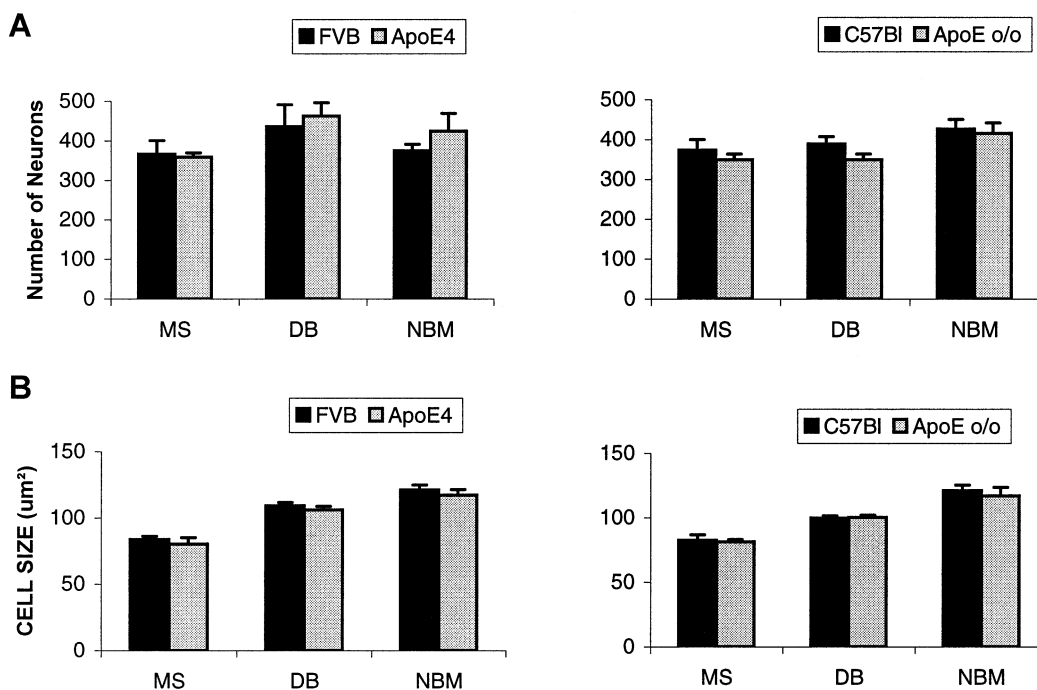


Fig. 2. Quantitative analysis of basal forebrain cholinergic neurons of apolipoprotein E4 transgenic, apolipoprotein E o/o, and non-transgenic mice. Size (A) and number (B) of ChAT-immunoreactive neurons in the medial septum (MS), horizontal limb of the diagonal band (DB) and nucleus basalis magnocellularis (NBM) of ApoE4 transgenic and ApoE o/o mice (grey bars) and non-transgenic mice (black bars). *n* = 4.

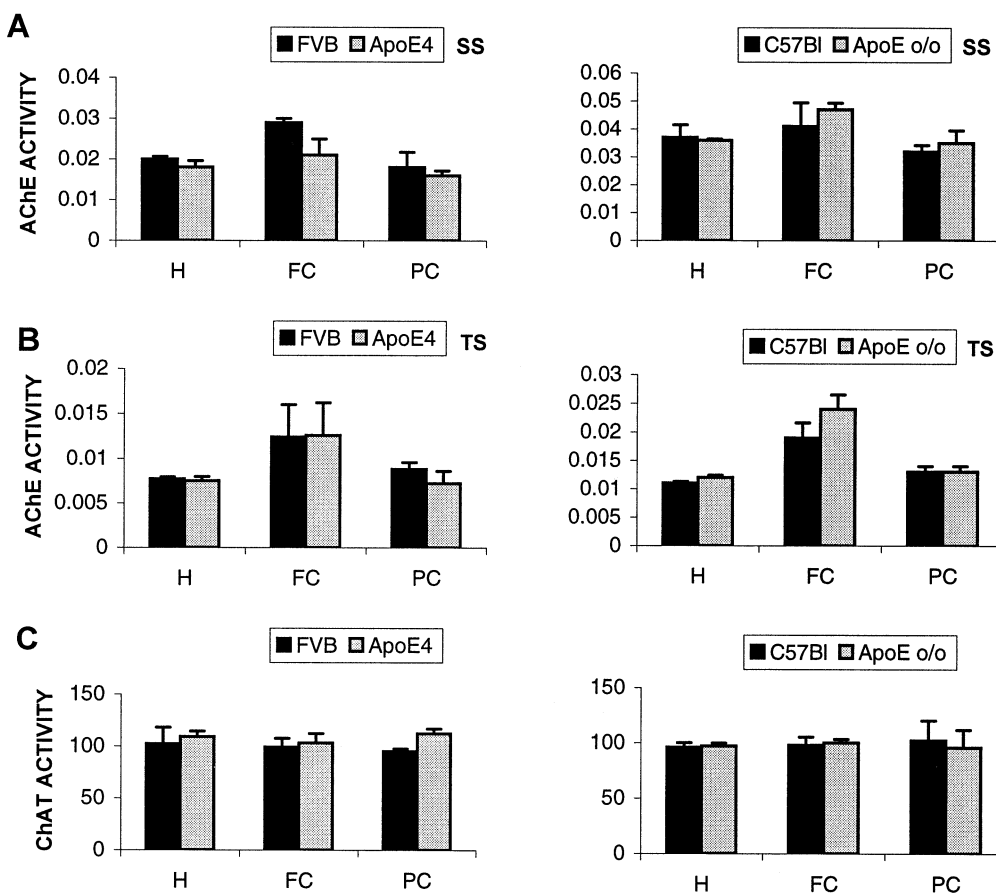


Fig. 3. Acetylcholinesterase and choline acetyltransferase activity in hippocampus (H), frontal cortex (FC), and parietal cortex (PC) of apolipoprotein E4 transgenic, apolipoprotein E o/o, and non-transgenic mice. A: Acetylcholinesterase activity in the salt soluble fraction (SS) from apolipoprotein E4 transgenic and apolipoprotein E o/o mice (grey bars) and non-transgenic mice (black bars). B: Acetylcholinesterase activity in the Triton soluble fraction (TS) from apolipoprotein E4 transgenic and apolipoprotein E o/o mice (grey bars) and non-transgenic mice (black bars). C: Choline acetyltransferase activity from apolipoprotein E4 transgenic and apolipoprotein E o/o mice (grey bars) and non-transgenic mice (black bars), in salt and Triton soluble fractions combined. Each data point is the mean of determinations on four individual mice of each genetic make-up.

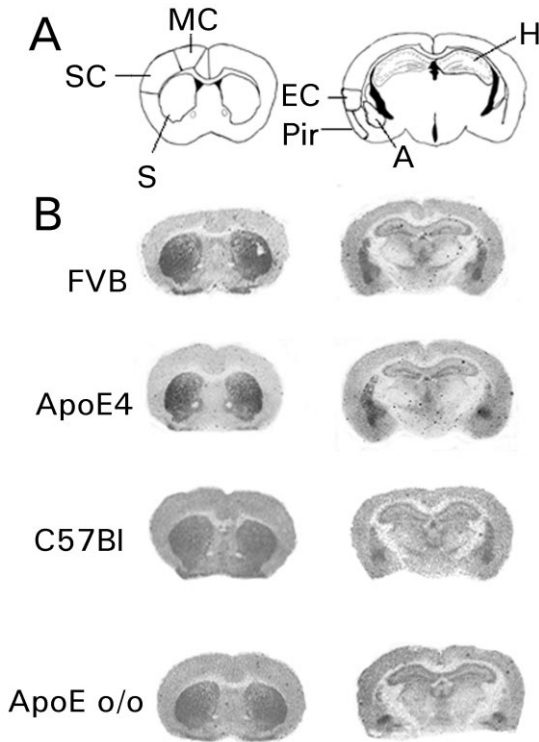


Fig. 4. Autoradiograms of [<sup>3</sup>H]hemicholinium-3 binding sites of apolipoprotein E4 transgenic, apolipoprotein E o/o and non-transgenic mice. A: Schematic representation of a mouse brain section through the striatum (left) and through the hippocampus (right). The brain areas used for the quantitative determination of  $K_d$  (dissociation constant) and  $B_{max}$  (maximal binding sites) (shown in Table 1) are indicated. MC, motor cortex, SC, somatosensory cortex, Pir, piriform cortex, EC, entorhinal cortex, H, hippocampus, A, amygdala, S, striatum. B: Representative autoradiograms from sections through the striatum (left) and through the hippocampus (right) are shown for ApoE4 transgenic, ApoE o/o, and non-transgenic mice as indicated.

station AIS/C. The optical density value from the non-specific binding was subtracted from each of the values. Each brain region was measured bilaterally in one section per concentration for each of the mice analysed (mice of each genotype). The  $K_d$  and the  $B_{max}$  were obtained by modified Scatchard analysis of [<sup>3</sup>H]hemicholinium-3 binding curves.<sup>45</sup>

#### Statistics

Student's two-tailed *t*-test was used to determine the statistical significance ( $P < 0.05$ ). The averages are expressed as the mean  $\pm$  S.E.M.

### RESULTS

The cholinergic cells, in mammalian brain located in several nuclei of the basal forebrain, are defined as ChAT-immunoreactive neurons. These nuclei do not have clear boundaries and are intermingled with non-cholinergic cells.<sup>20</sup> We have mapped these nuclei in the brain of non-transgenic mice from the FVB and the C57Bl/6 mouse strains, which were used throughout this study as controls. In both strains the cholinergic structures were anatomically similar. No evidence for reduced cholinergic cell size or morphological alterations was observed in any of the transgenic mice analysed, i.e. ApoE4 transgenic mice or ApoE o/o mice (Figs 1A–H and 2A). Furthermore, the number of cholinergic cells measured in age-matched non-transgenic mice and in transgenic mice was not significantly different (Fig. 2B). AChE enzymatic

Table 1. Quantitative analysis of [<sup>3</sup>H]hemicholinium-3 binding in brain of apolipoprotein E4 transgenic mice, apolipoprotein E o/o mice and age-matched non-transgenic mice

	FVB		ApoE4		C54Bl		ApoE o/o	
	$K_d$ (nM)	$B_{max}$ (fmol/mg tissue)	$K_d$ (nM)	$B_{max}$ (fmol/mg tissue)	$K_d$ (nM)	$B_{max}$ (fmol/mg tissue)	$K_d$ (nM)	$B_{max}$ (fmol/mg tissue)
Motor cortex (MC)	6 $\pm$ 1	97 $\pm$ 19	8 $\pm$ 1	78 $\pm$ 11	10 $\pm$ 2	87 $\pm$ 14	10 $\pm$ 3	110 $\pm$ 21
Somatosensory cortex (SC)	9 $\pm$ 3	103 $\pm$ 18	8 $\pm$ 1	78 $\pm$ 12	10 $\pm$ 1	97 $\pm$ 12	10 $\pm$ 1	52 $\pm$ 3
Piriform cortex (Pir)	5 $\pm$ 0.3	116 $\pm$ 21	6 $\pm$ 1	82 $\pm$ 10	9 $\pm$ 0.5	78 $\pm$ 6	8 $\pm$ 0.6	71 $\pm$ 4
Entorhinal cortex (EC)	6 $\pm$ 0.2	124 $\pm$ 25	7 $\pm$ 1	81 $\pm$ 10	10 $\pm$ 2	92 $\pm$ 9	10 $\pm$ 2	94 $\pm$ 8
Hippocampus (H)	6 $\pm$ 0.6	120 $\pm$ 20	5 $\pm$ 1	82 $\pm$ 10	13 $\pm$ 3	110 $\pm$ 11	10 $\pm$ 3	97 $\pm$ 15
Amygdala (A)	6 $\pm$ 1	300 $\pm$ 27	5 $\pm$ 0.7	266 $\pm$ 23	9 $\pm$ 1	298 $\pm$ 37	11 $\pm$ 3	330 $\pm$ 41
Striatum (S)	4 $\pm$ 0.5	341 $\pm$ 22	6 $\pm$ 1	374 $\pm$ 11	7 $\pm$ 1	348 $\pm$ 32	4 $\pm$ 0.4	275 $\pm$ 24

Brain sections were incubated with six different concentrations (1.25–30 nM) of [<sup>3</sup>H]hemicholinium-3 (120 Ci/mmol). Autoradiographic images from the sections were scanned and the optical density in the different brain areas illustrated in Fig. 4 were quantified and standardized with values of the tritium microscale standards (fmol/mg of tissue). Scatchard analysis of the binding curves allowed the calculation of the dissociation constant ( $K_d$ ) and the maximum binding sites ( $B_{max}$ ) per region ( $n = 4$ , mean  $\pm$  S.E.M.). Differences, analysed by Student's *t*-test ( $P < 0.05$ ), were not significant.

activity co-localizes with ChAT in mouse brain, indicating that AChE histochemistry is a reliable marker for cholinergic fibers.<sup>28</sup> In none of the transgenic mice did AChE enzymatic activity histochemistry reveal major changes in the pattern of fiber distribution in the hippocampus (Fig. 1 I–L) and frontal cortex (Fig. 1 M–P).

The AChE activity (in salt and in Triton brain extracts) and the ChAT activity were not different in the frontal cortex, in the parietal cortex and in the hippocampus of ApoE4 transgenic mice and ApoE o/o mice compared to age-matched non-transgenic mice (Fig. 3).

Hemicholinium-3 is a specific ligand for high affinity choline transporter (HACHT) in rodent brain and was used as a reliable marker for cholinergic terminals.<sup>25</sup> The regional distribution of [<sup>3</sup>H]hemicholinium-3 binding sites was analysed in the brain of ApoE4 transgenic mice, ApoE o/o mice and age-matched non-transgenic mice. Neither the qualitative (Fig. 4) nor the quantitative data from selected brain regions (Table 1) revealed changes in the distribution, in the maximum number of binding sites ( $B_{\max}$ ) and in the dissociation constant ( $K_d$ ) in ApoE4 transgenic mice and ApoE o/o mice compared to age-matched non-transgenic mice (Fig. 4; Table 1).

#### DISCUSSION

We have undertaken an extensive analysis of cholinergic parameters in mice overexpressing the human ApoE 4 protein and in ApoE knockout mice with the objective to analyse whether these mice develop cholinergic problems. The parameters analysed were chosen because they are compromised in AD brain.<sup>32,9,5,56</sup> The limiting step for the synthesis of acetylcholine is the choline transport.<sup>25</sup> The high affinity choline transporter is particularly interesting because in addition to being a well-known presynaptic cholinergic marker, the level of the transporter is regulated by the activity of the cholinergic neurons, thereby constituting a functional marker of cholinergic synapses. The rationale to perform this study was the reported increased severity of cholinergic problems in AD

patients carrying  $\epsilon 4$  alleles.<sup>52,40,3,7,47</sup> Even the presence of one ApoE4 allele appears sufficient to induce cholinergic deleterious effects in normal subjects,<sup>3</sup> suggesting that the ApoE4 isoform could exert a chronic effect on the cholinergic circuitry.

The outcome of our studies indicated that no cholinergic malfunction was detected, either in transgenic mice overexpressing the human ApoE4 isoform or in ApoE deficient mice. ApoE null mice have been proposed as a suitable mouse model for cholinergic hypofunction.<sup>22</sup> This proposition was not supported either by our extensive quantitative experimental work or by the results of other groups in other mouse strains.<sup>4,16,43,30</sup>

The present results provide a solid quantitative and qualitative basis to conclude against the proposition that ApoE is absolutely necessary for maintaining normal functioning of the cholinergic system. It is also possible that other proteins compensate for the function of ApoE. In addition, we demonstrate for the first time that overexpression of human ApoE4 in mouse brain is not *per se* deleterious for the cholinergic system. The poor outcome of AD patients carrying the ApoE4 allele to cholinergic therapy and the presence of stronger cholinergic deficit in those patients may be due to a general impairment in the neuronal remodeling process. In addition, the increased load of amyloid plaques and neurofibrillary tangles in AD patients is modulated by the presence of the  $\epsilon 4$  allele.<sup>51,35,7</sup> Since the load of amyloid plaques and neurofibrillary tangles in AD brain correlates with the severity of the cholinergic deficit, the effect of the ApoE4 isoform on the cholinergic system could be indirect.<sup>38,48,6</sup> It has been reported that carriers of  $\epsilon 4$  alleles have a poor outcome after head injury and brain stress, although no molecular data are available.<sup>2,46</sup> Possibly, the influence of the allelic variants of ApoE on the cholinergic problems might become evident only after the cholinergic system has been damaged. This possibility, which would be an indirect effect, remains to be tested in our ApoE4 and ApoE knockout mice. Our results exclude a direct toxic effect of the ApoE4 isoform on the cholinergic system in mouse brain, in the absence of other insults.

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(Accepted 10 January 2000)