Molecular Psychiatry (2014) 19, 1243–1250

www.nature.com/mp

ORIGINAL ARTICLE

APOE2 enhances neuroprotection against Alzheimer's disease through multiple molecular mechanisms

C Conejero-Goldberg[1], JJ Gomar[1,2,5], T Bobes-Bascaran[1,5], TM Hyde[3], JE Kleinman[3], MM Herman[4], S Chen[1], P Davies[1] and TE Goldberg[1]

The common APOE2 gene variant is neuroprotective against Alzheimer's disease (AD) and reduces risk by nearly 50%. However, the mechanisms by which APOE2 confers neuroprotection are largely unknown. Here we showed that ApoE protein abundance in human postmortem cortex follows an isoform-dependent pattern (E2>E3>E4). We also identified a unique downstream transcriptional profile determined by microarray and characterized by downregulation of long-term potentiation (LTP) related transcripts and upregulation of extracellular matrix (ECM)/integrin-related transcripts in E2 cases and corroborated this finding at the protein level by demonstrating increases in ECM collagens and laminins. *In vivo* studies of healthy older individuals demonstrated a unique and advantageous biomarker signature in E2 carriers. APOE2 also reduced the risk of mild cognitive impairment to AD conversion by half. Our findings suggest that ApoE2 protein abundance, coupled with its inability to bind to LDLRs, may act to increase amyloid-beta (Ab) clearance. In addition, increased ECM and reduced LTP-related expression results in diminished activity-dependent Ab secretion and/or excitotoxicity, and thus also promotes neuroprotection.

Molecular Psychiatry (2014) **19,** 1243–1250; doi:10.1038/mp.2013.194; published online 4 February 2014

Keywords: Alzheimer's disease; APOE2; biomarkers; gene expression; human brain; neuroprotection

INTRODUCTION

The APOE gene is triallelic at two SNPs in its exon 4 (rs429358, rs7412), resulting in amino acid substitutions of arginine or cysteine at positions 112 and 158. E2 corresponds to Cys/Cys, E3 to Cys112/Arg158, and E4 to Arg/Arg.[1,2] Although E4 is the major genetic risk variant for late onset AD (OR = 3.81), the E2 variant is neuroprotective and reduces risk for AD by nearly 50% (OR = 0.54) when contrasted to the referent variant E3.[3,4] The allele frequency of APOE2 in the general Caucasian population is about 8%. In contrast, APOE2 may be modestly associated with cerebral amyloid angiopathy and has been inconsistently associated with risk for several other neurologic diseases.[5–9] APOE2 homozygosity is associated with type III hyperlipoproteinemia.[5,10] Nevertheless, APOE2 has been associated with reduced cardiovascular disease and greater longevity.[11–14]

ApoE is expressed primarily in astrocytes and delivers cholesterol and other lipids to neurons through the low-density lipoprotein receptor family (including LDLR, LRP1, VLDLR and ApoER2).[15,16] Several features of the E2 isoform distinguish it from the E3 and E4 isoforms. These include dramatically reduced binding to LDLRs in comparison to E3 and E4 and conformational stability in comparison to E4.[2,17] APOE isoforms differentially regulate products of amyloid precursor protein processing.[18,19] Some amyloid-beta (Ab) peptides, for example, Ab1–42, cleaved from amyloid precursor protein , are thought to be toxic and may have key roles in AD neurodegeneration. E2 and E3 bind to Ab with greater affinity than does E4, and so isoforms may differentially regulate Ab clearance through neurons, microglia or delivery to the blood-brain barrier (BBB).[20–22]

Despite the potential importance of APOE2, there have not been comprehensive studies of its molecular properties, downstream impact on biological pathways, modulation of brain function parameters and neurodiagnostic impact. Here we will examine these questions using molecular techniques to assess mRNA and protein levels of E2 in comparison to E3 and E4 in human postmortem neocortex, examine the transcriptional profile of E2 carrier cases, determine if an E2 signature is present in combined biomarker and cognitive analyses in healthy older individuals, and last, examine the effects of E2 on neurodiagnosis in the context of MCI to AD conversion.

MATERIALS AND METHODS

Study 1

Human Brain Samples. Fresh-frozen human tissue was obtained from the National Institute of Mental Health Clinical Brain Disorders Branch, GCAPP, Neuropathology Section. For all molecular studies, we studied younger cases (who were 60 years or less at death) and included 5 APOE2, 13 APOE3/E3, and 13 APOE4 carriers (two of whom were E4 homozygotes). Demographic characteristics of the postmortem cases are in Supplementary Table 1. All specimens underwent neuropathological examination, including Bielschowsky silver staining, for plaque and tangle density, vascular infarcts and other neurodegenerative conditions; they were free of diagnosable AD and CVD pathology. For each case, we collapsed assay results from tissue obtained from two cortical regions (BA 1/2/3 and BA 21). As noted, no case had diagnosable AD neuropathology. Further details about the samples can be found in Conejero-Goldberg *et al.*[23] DNA from brain tissue of the above cases was used for genotyping.[16] The APOE genotype groups did not

[1]Litwin Zucker Center for the Study of Alzheimer's Disease, Feinstein Institute, Hofstra North Shore-LIJ School of Medicine, Manhasset, NY, USA; [2]FIDMAG, Hermanas Hospitalarias, Sant Boi de Llobregat, Spain; [3]Neuropathology Section, Lieber Institute for Brain Development, Baltimore, MD, USA and [4]Section on Neuropathology, Clinical Brain Disorders Branch, GCAPP, IRP, National Institute of Mental Health, NIH, Bethesda, MD, USA. Correspondence: Dr TE Goldberg, Litwin Zucker Center for the Study of Alzheimer's Disease, Feinstein Institute, Hofstra North Shore-LIJ School of Medicine, Manhasset, NY 11030, USA.
E-mail: tgoldber@nshs.edu
[5]These authors contributed equally to this work.
Received 3 April 2013; revised 13 November 2013; accepted 16 December 2013; published online 4 February 2014

differ significantly in demographic or tissue quality variables as shown in Supplementary Table 1.

RT-qPCR. The RT-qPCR reactions were carried out in an ABI Prism 7900HT thermal cycler to determine the ΔΔCt as described previously.[23]

Immunoassays. More detailed information about methods of supernatant collection and western blotting is in Supplementary Methods. *Western Blotting*. Blots were probed with rabbit anti-ApoE (H-223) polyclonal antibody (1:400 v/v dilution) and mouse anti- β-actin monoclonal antibody (1:2000 v/v dilution) (Santa Cruz Biotechnology, CA, USA). Detection was done using enhanced chemiluminiscence (ECL, GE Healthcare Life Sciences, Pittsburgh, PA, USA). Signal intensity was quantified using NIH ImageJ software. We assessed protein levels of ApoE isoforms (at 34–39 kDa) and assessed proteolytic fragments (at 17–22 kDa), insofar as they were present/absent. *ELISA*. Levels of human APOE in brain were measured by human-specific APOE ELISA (MBL International, Woburn, MA, USA) according to the manufacturer's instructions. Diluted homogenates were run in triplicate and detected using Pan-APOE antibody in a TECAN Genios Pro Microplate Reader. All APOE measurements were normalized to total protein in the brain homogenates; ELISA values were expressed as ng ml^{-1}.

Statistical Approach. We utilized an analytic strategy that examined APOE genotype differences in a GLM framework (SAS Proc GLM) with reference molecules/gene products serving as covariates as necessary, followed by planned contrasts in which E2 samples were compared to E3 samples and to E4 samples. Using western blot data, we also analyzed the number of cases that demonstrated proteolytic fragments and compared the distribution by Fisher's test across genotype groups. Here and elsewhere in the report, we studied E2/E3 cases (hereafter E2), E3/E3 (hereafter E3) and E4 carriers (hereafter E4) in human samples or humans. E2/E4 cases were excluded. E3/E3 cases were considered the referent variant (as by convention) for the purposes of this study.

Study 2

Human Brain Samples. As detailed above, with the exception that we included 11 additional E3 cases. These cases did not show substantively different tissue quality or demographics from those cases listed in Supplementary Table 1. We utilized only tissue from BA 21 in this experiment.

Microarray Platform. The platform used was the WG6 BeadChip array (Illumina, San Diego, CA, USA), generating whole-genome expression profiles for six samples in parallel on a single array.

Data pre-processing and analysis. The data were pre-processed using BeadStudio software (Illumina). Technical replicates of the same sample were averaged after it was determined that correlations between the replicates were consistently very high (>0.98). We identified genes that were differentially expressed among the two classes (APOE3-carriers/APOE2-carriers) computing an ANOVA blocked by age and sex. A stringent significance threshold was used to limit the number of false-positive findings. In comparing the APOE3/E2 types, we also controlled for the age and sex of the patient.

RT-qPCR. For selected genes showing differential expression between APOE2 and APOE3 cases, cDNA synthesis was generated for each sample, and we determined the ΔΔCt and significance of the reported cases-control differences.

Immunoassay (Western Blotting). For laminin and collagen VI, blots were probed with rabbit anti-laminin polyclonal antibody (1:500 v/v dilution; Sigma-Aldrich, St Louis, MO, USA) and rabbit anti-collagen VI polyclonal antibody (1:1000 v/v dilution; Abcam, Cambridge, MA, USA). More information is in Supplementary Methods.

Study 3

Methods used in ADNI, including subject selection, biomarker assays, imaging parameters and cognitive testing, can be found on the ADNI website and in our previous work.[24] *Subjects*. We utilized 197 subjects who met ADNI inclusion criteria for HCs.[17] *APOE* genotype was available in the ADNI database. Twenty-four subjects were E2s (including 1 homozygote), 107 were E3/E3 carriers, and 47 were E4 carriers (including 3 homozygotes). For the cluster analysis, in which only subjects whose cognitive and biomarker data sets were complete were included, seventeen subjects were E2s, 61 were E3/E3s and 21 were E4 carriers.

Cognitive Tests. We analyzed data from the following tests: WMS-R Logical Memory delay; Auditory Verbal Learning Test Trial 5 score, Delay Score (free recall), trails b.

Biomarkers. *Structural MRI*. We used the Dale semi-automated method (FreeSurfer) for determining cortical thickness (middle temporal lobe), hippocampal volume, entorhinal thickness and total brain volume.[24] *CSF Ab1–42 and Tau*. We examined CSF Abeta and total tau and phospho tau levels in ADNI healthy control samples that were collected at baseline ('UPENNBIOMK'). Concentrations were log transformed for analysis.

Statistical Approaches. We first conducted GLM-based procedures to determine if differences among the APOE genotype groups were present at baseline. We then conducted an unsupervised cluster analysis (PROC CLUSTER Method = AVE in SAS) which we considered central to identifying an E2 'signature,' that is, the presence of clusters of cases (subjects) on the basis of cognitive and biomarker level and profile. APOE genotype was excluded from these analyses, so that clusters could be aggregated independent of genetic information. After identification of clusters, we then examined whether any one cluster contained a disproportionate number of E2 carriers by X^2.

Study 4

Subjects. The ADNI aMCI sample of 310 patients was followed for more than 4 years. Criteria for MCI were the same as defined by Petersen and outlined in Leduc *et al*.[17] Fifteen patients were carriers of the APOE2 allele, 128 patients were E3 homozygotes and 167 patients were APOE4 carriers (including 41 E4 homozygotes). Demographic and brain volumetric variables are reported in Supplementary Table 7.

Statistical Approaches. We utilized Cox Proportional Hazard Regression (HR) models in order to determine the unique multiplicative effect of genotype differences (that is, a 'unit increase') on hazard rate (conversion from MCI to AD).

RESULTS

Study 1. Molecular properties of ApoE isoforms

mRNA and Protein Levels. Protein levels may offer a window to partially understand protective effects as ApoE protein level may be an important determinant of efficient and robust lipid transport, thought to be necessary for synaptic growth, remodeling and repair, and for Ab clearance.[17,25] It has also been suggested that protein fragments are cleaved differentially among isoforms in neurons, with toxic downstream consequences including mitochondrial dysfunction most frequent in APOE4.[26,27] E2 protein levels and fragmentation have not been examined in human postmortem cortical samples. In contrast, in human plasma, there are well-replicated differences in protein level, such that E2>E3>E4 (for example, Soares *et al*.[28]).

We first sought to determine if APOE genotype differences in mRNA expression were present in human postmortem cortex. We then examined protein level and tendency toward fragmentation as a function of APOE genotype in these samples (see Supplementary Table 1 for demographics and tissue quality of cases). In transgenic mice with human APOE gene variants knocked-in, differences between E2, E3 and E4 mRNA isoform levels were not found.[29,30]

mRNA Expression. Using RT-qPCR, we compared ApoE expression in the three genotype groups (E2/E3, E3/E3, E4 carriers), and we did not find significant isoform-related differences.

Immunoassays. Using a polyclonal antibody for ApoE, we compared protein levels of E2, E3 and E4 cases, and identified

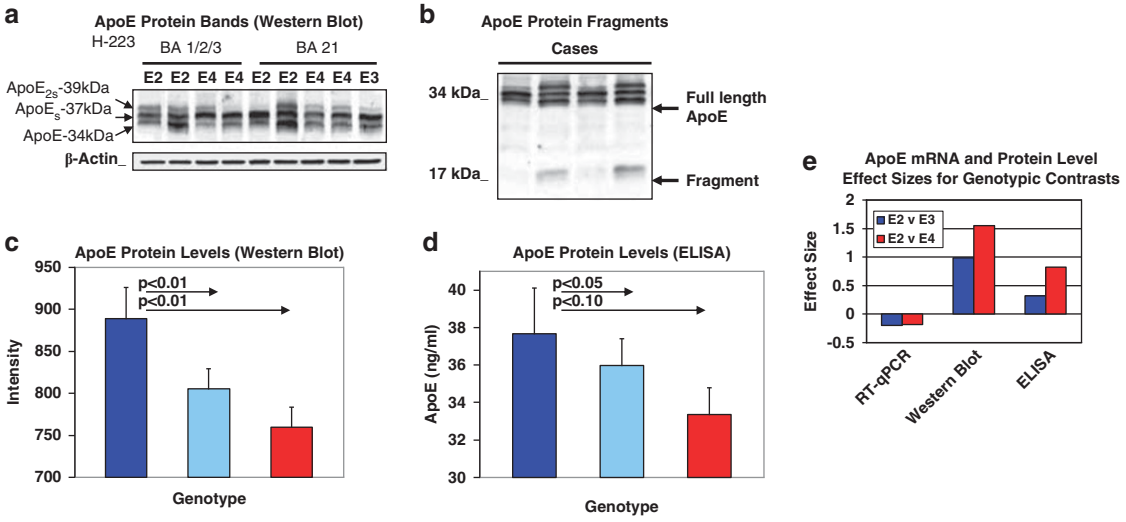

Figure 1. ApoE protein level in human brain from APOE2, APOE3 and APOE4 carriers. (**a**) Western blot analysis showed ApoE protein expression in human brain from APOE2, APOE3 and APOE4 carriers in two brain areas: BA 1/2/3 and BA 21 using a polyclonal antibody for ApoE (H-223). Immunoreactive 34-kDa native ApoE and 37-kDa and 39-kDa sialylated ApoE (ApoEs and ApoE2s) bands were observed in E2, E3 and E4 carriers. (**b**) ApoE fragments in human cortex in normal control brains. Immunoreactive 34-kDa native ApoE and 37-kDa and 39-kDa sialylated ApoE (ApoEs and ApoE2s) bands were observed along with a 17-kDa fragment using a polyclonal antibody for ApoE (H-223). (**c**) Protein levels of E2, E3 and E4 carriers in brain tissue were compared. For the polyclonal immunoassay measuring differences in ApoE protein, a GLM analysis of covariance (ANCOVA) (beta-actin corrected) with planned contrasts demonstrated an overall significant difference among the genotype groups that was significant ($F2,1,1 = 6.44$, $P = 0.005$) for combined bands. The E2-E3 contrast was at a trend level for significance ($F = 3.50$, $P = 0.07$); the E2-E4 contrast was significant ($F = 8.52$, $P = 0.007$). (**d**) By ELISA a trend level difference among ApoE isoforms was present ($F2,1,1 = 2.67$, $P = 0.08$). In planned contrasts the E2-E3 difference was significant ($F = 4.17$, $P = 0.05$); the E2-E4 difference was significant at a trend level ($F = 2.82$, $P = 0.10$). (**e**) Effect size differences (Cohen's d) for RT-qPCR (for which no genotype differences were found), polyclonal, and ELISA values for E2 vs E3 differences and E2 vs E4 differences.

three bands by western blot: band 1 at 39-kDa, band 2 at 37-kDa and band 3 at 34-kDa (Figure 1a). The band with the highest molecular weight (band 1) is thought to reflect heavily sialylated ApoE; 34 kDa ApoE is thought to reflect the native species.[31] We also identified 17–22 kDa ApoE that reflects protein fragments (Figure 1b). Total protein differed significantly among the genotype groups (E2>E3>E4) as described in Figure 1c. Differences were largest in the native ApoE and lightly sialylated band (data not shown). By ELISA, the overall difference in protein level among the APOE genotype groups approached significance (Figure 1d). Western blot-based fragmentation rates did not differ among genotypes by Fisher's exact test. Numerically, E2 had the lowest proportion of fragments (see Supplementary Table 2).

Effect Sizes. For mRNA, effect sizes for E2-E3 and E2-E4 contrasts were small as shown in Figure 1e. For polyclonal-based measures of protein abundance, effect sizes shown in Figure 1e were large in the E2-E3 and E2-E4 contrasts. For ELISA-based contrasts, effect sizes were medium for E2-E3 and E2-E4 contrasts as in Figure 1e.

Summary. We found a mismatch between mRNA expression levels and protein levels in APOE genotype contrasts. While mRNA expression levels did not differ, protein levels were higher in E2 cases, suggesting that the isoform was relatively impervious to post-translational modifications and in keeping with several *in vivo* and *in vitro* studies that have implicated differences in proteolytic degradation among the isoforms.[2,17] In particular, in several studies of APOE targeted replacement (TR) transgenic mice, mRNA expression levels did not differ among APOE variants, but protein levels were lowest in E4 and highest in E2 mice.[29,30] Pulse chase experiments in astrocyte-derived cell cultures have indicated enhanced degradation of newly synthesized E4 protein.[29] Associated with these differences in the AD transgenic mice were differences in brain amyloid burden, such that E2 mice had the lowest level of amyloid and E4 mice had the highest level. Thus, this pattern of findings in human brain recapitulates with great fidelity several transgenic animal studies using human APOE TR.

Study 2. Transcriptional profiling of APOE2 in human cortex

In order to examine downstream consequences of APOE2 on the transcriptome, we conducted a microarray study that contrasted the transcriptional profiles of E2 and E3 carrier cases in human postmortem cortex. To the best of our knowledge there have been no prior studies in this area. We sought to identify key biologic pathways that might plausibly confer neuroprotection or enhanced neuronal function.

Group Comparison of Transcription Profiles. We identified 131 transcripts that differed significantly (at $P < 0.001$) between APOE2 and APOE3-carriers using ANOVA for fixed effects in the BRB analysis suite. A large proportion of these individual transcripts (73%) were upregulated in E2 carriers with respect to E3 carriers. Results are listed in Supplementary Table 3.

Hierarchical Clustering of Expression Changes Identified by ANOVA. For the hierarchical clustering only, we excluded the 16 LOC genes, 43 ESTs and 6 open reading frames that have been identified informatically from the human transcriptome, leaving a total of 66 transcripts for further analysis, of which 41 were upregulated.

As shown in the heat map image (Figure 2), the hierarchical clustering of expression changes derived from these transcripts demonstrated a cluster of four cases in the APOE2 group. The APOE3 group demonstrated a less distinct pattern of transcriptional upregulation and downregulation.

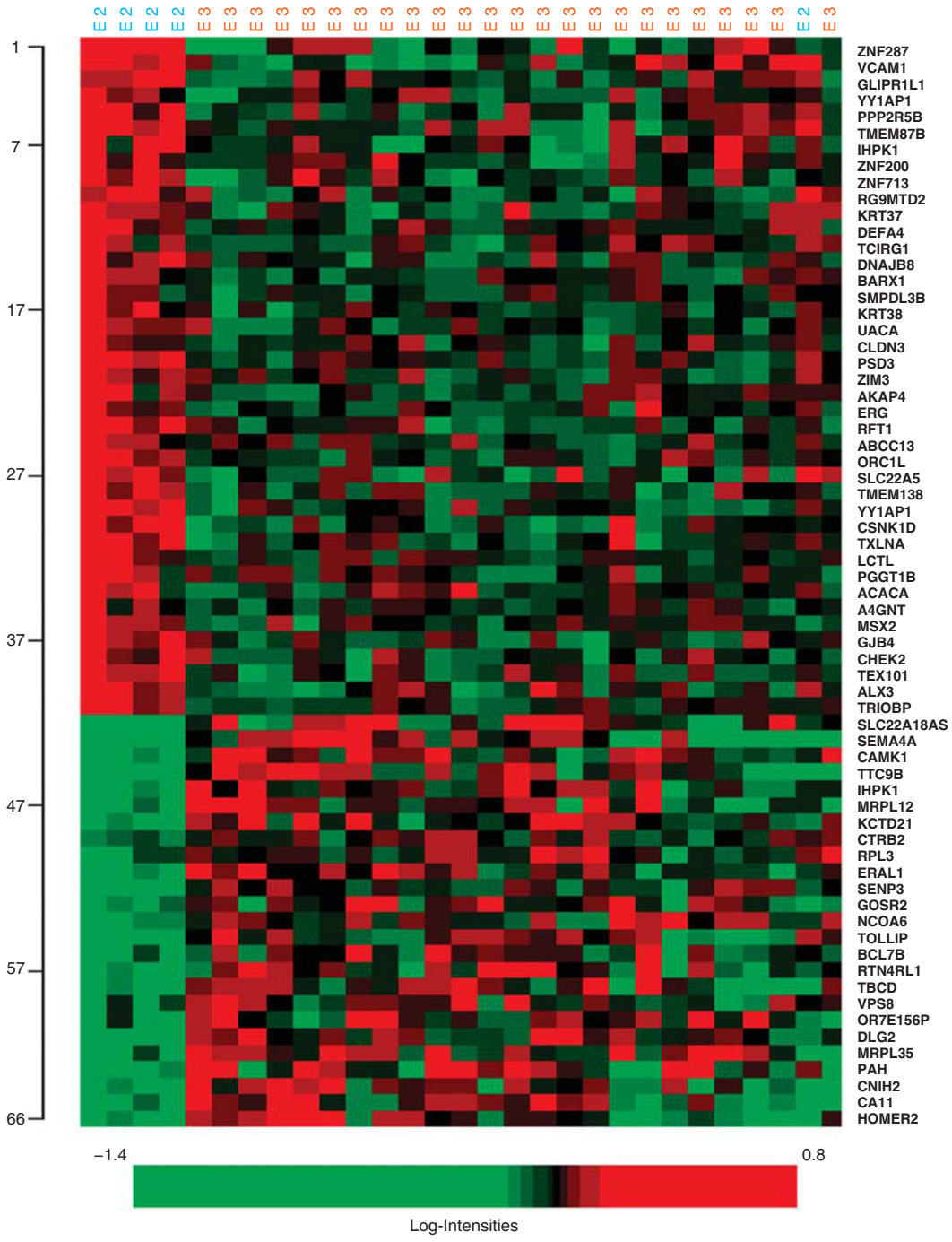

Figure 2. Heat map showing up- and down-regulations on a case-by-case basis for the 66 transcripts that were identified as significant by analysis of variance (ANOVA) analysis (all *Ps* < 0.001). Transcripts are on the *y* axis, and cases grouped according to their similarity to their neighbors are on the *x* axis. Log intensities are represented in colored cells. Red cells indicate relatively strong upregulation in BA 21 (temporal cortex); while green cells indicate the converse. Forty-one transcripts were upregulated in the E2 carriers as compared with E3 carriers; 25 transcripts were downregulated.

CypA. In addition, based on recent work[32] identifying Cyclophilin A (CypA) as an important mediator of BBB integrity and differentially modulated by ApoE isoforms, we examined its expression *post hoc*. We found that CypA mRNA was reduced in E2 carrier group ($P = 0.01$), in keeping with the view that E2 is associated with more robust clearance of Ab at the perivasculature.[33]

Biological and Signaling Pathways Analysis. Two Kyoto pathways were identified with LS/KS and Efron-Tibshirani's GSA test using *P*-values of $P < 0.005$: the long-term potentiation (LTP) and the extracellular matrix (ECM)-receptor interaction.[34,35] Genes within these pathways are listed in Supplementary Tables 4(a-b).

RT-qPCR. We examined transcripts related to LTP (HOMER2 and DLG2) and the ECM (COL6A1, LAMC1, ITGB1) by RT-qPCR. We validated the microarray results by RT-qPCR in four genes (HOMER2, DLG2, COL6A1, LAMC1, all *Ps* < 0.05 by *t*-test). See

Supplementary Table 5 for fold changes and comparison of microarray probe to RT-qPCR data and Supplementary Results for a brief overview of the molecules in question.

To further extend these results, we also conducted Western blot studies to assay levels of select ECM related proteins in the E2 group. Using a polyclonal antibody for laminin, we found a highly significant increase in protein level in the E2 group contrasted with the E3 group. Using a polyclonal for collagen VI, we also found a highly significant increase in protein level in the E2 group. Details of the analyses are in Supplementary Methods and Supplementary Figure 1(a-b).

Summary. In addition to multiple individual transcripts that differed between the E2 and E3 groups, we identified two pathways that may protect neurons in the E2 group. They involved LTP and ECM. E2 cases had lower expression levels of transcripts associated with LTP. This is in keeping with prior work in APOE TR mice that demonstrated that E2 carriers showed reduced LTP in comparison to other APOE genotypes.[36] Because of findings of hypermetabolism in nodes of the so-called brain default network, activity-dependent Ab deposition, and excitotoxicity,[37–39] reductions in LTP-related activity may be protective in the E2 context. Similarly, a recent study demonstrated that brain activity may be related to DNA double-strand break neurodegeneration and that such degeneration worsened in the presence of Abeta.[40] Reductions of activity might also attenuate this type of neurodegenerative process. Furthermore, our findings of ECM integrin-related upregulations in E2 carriers serve to provide a mechanism for activity reduction. Increases in the ECM reduce plasticity, perhaps specifically by impacting motility of AMPA receptors, crucial for early phase LTP.[41,42] As noted, insofar as Ab is secreted in an activity-dependent manner, reduced LTP might attenuate Ab levels. In addition, increases in the ECM ('perineural nets') at synapses reduce tangle formation in situ.[43]

Another neuroprotective mechanism may involve our finding of lowered CypA expression; reductions of this are thought to have positive consequences for Ab clearance across the BBB.[33] ECM collagen and laminin increases (as found in the protein experiments described above) may also promote BBB integrity.[44]

Interestingly, the pathways did not overlap with the signaling pathways and biological processes associated with E4 in our earlier microarray study.[23] We thus suggest that the pathways toward neurodegeneration and neuroprotection are not simply the inverse of one another.

Study 3. Biomarker profile of APOE2

We sought to determine if E2 carriers had better neurocognition and more advantageous biomarker profiles.[45–50] We used the ADNI public database because of the large samples of healthy controls (HCs) (mean age 75 years), APOE characterization, reasonable numbers of E2/E3 carriers in the HC group in conformity with population allele frequencies and the wide range of cognitive markers and biomarkers collected in a uniform and prospective manner.

Genotype Differences in Biomarkers. We first compared the APOE genotype groups on a panel of biomarkers. Significant differences were present for CSF Ab1–42, p-tau, and middle temporal cortical thickness (Table 1). Effect sizes were large to moderate (Figure 3a). No significant differences were present for cognitive markers (Table 1); effect sizes were generally small (Figure 3b).

We next conducted a cluster analysis. In a combined panel of cognitive and biomarkers, we identified a robust solution indicating the presence of three clusters (cubic clustering criterion = 1.71, $R^2 = 0.65$, estimated $r^2 = 0.61$, pseudo-$F = 89.00$). Nine cases were in cluster 1, 59 in cluster 2, and 31 in cluster 3 (Figure 3c). As noted, APOE genotype was not utilized as a clustering variable. We then interrogated each of the aggregates in order to determine their respective proportion of E2, E3 and E4 cases. Genotypes were unequally distributed by Fisher's exact test (see Supplementary Table 6). Cluster 2 had the highest proportion of E2 genotypes (15/17) and lowest of E4 genotypes (5/21). We also examined *Z*-score profiles of the clusters (based on the grand means/variance of the healthy control group). Cluster 2 included nearly every E2 case, a majority of E3 cases, and relatively few E4 cases, and was associated with an advantageous biomarker profile (high Ab, low p-tau, increased middle temporal cortical thickness, modestly higher cognitive scores) compared with clusters 1 and 3.

Table 1. Comparison in healthy controls of APOE genotype groups on cognitive and biomarkers by ANOVA, followed by planned contrasts when overall *F* and *P* were significant

Test	*df*	F	P	*E2 vs E3* (F, P)	*E2 vs E4* (F, P)
MMSE	2,194	1.21	0.30		
Left hippocampus	2,191	0.43	0.65		
Left entorhinal	2,191	1.53	0.22		
Left middle temporal cortex	2,191	3.80	0.02	3.35, 0.06	7.51, 0.006
Ab1–42	2,98	13.40	0.0001	7.93, 0.005	26.37, 0.0001
T tau	2,98	1.21	0.30		
P tau	2,88	3.40	0.04	2.01, 0.05	2.39, 0.02
AVLT	2,196	0.02	0.89		
Logical memory	2,196	0.19	0.82		
Semantic fluency	2,196	2.02	0.14		
Clock test		0.27	0.76		
Trails	2,194	0.78	0.46		

Abbreviations: AVLT, auditory verbal learning test; MMSE, mini-mental state examination.

Summary. APOE2 was associated with dramatically increased CSF Ab and lower p-tau. In models of neurodegeneration, low-CSF Ab are considered to be an early 'preclinical' phase indicator of increased risk for AD pathogenesis.[51] In principle, high Ab associated with E2 status should be associated with reduced risk. APOE2 also reduced p-tau (but not t-tau), suggesting that it had a specific role in reducing phosphorylation at threonine 181. An E2 signature was identified and derived independent of genotype in an unsupervised cluster analysis in healthy older individuals. Cluster 2, which contained the majority of E2 carriers, reflected an *in vivo* version of exceptional aging and a protective profile that is quite dissimilar to the MCI at risk or AD biomarker profiles described in this sample and in other work.[24,52]

Study 4. APOE2 and risk of MCI to AD conversion. We next sought to determine if the E2 genotype had clinical and neurodiagnostic consequences. While it is known that E2 reduces the probability of incident AD, it is unknown if E2 can delay or reduce the risk of dementia, even in those individuals with a diagnosable amnestic memory impairment (that is, amnestic MCI). We examined the proportion of E2 cases in MCI to AD converters and nonconverters (in contrast to E3 and E4 genotypes) in an ADNI sample in Cox regression hazard analyses over a 4-year period.

Cox Regression Analyses. During follow-up, 3 (20%) of MCI E2 carriers, 46 (35.9%) of MCI E3, and 96 (57.5%) of MCI E4 carriers were uncensored (developed AD). The mean follow-up time in months for MCI APOE E2 carriers who developed AD was 34.18 (range 24.31–41.36; s.d. = 8.84), for MCI APOE E3 homozygotes was 18.59 (range 5.75–42.25; s.d. = 9.38), and for MCI APOE E4 carriers was 20.29 (range 5.98–52.63; s.d. = 10.84).

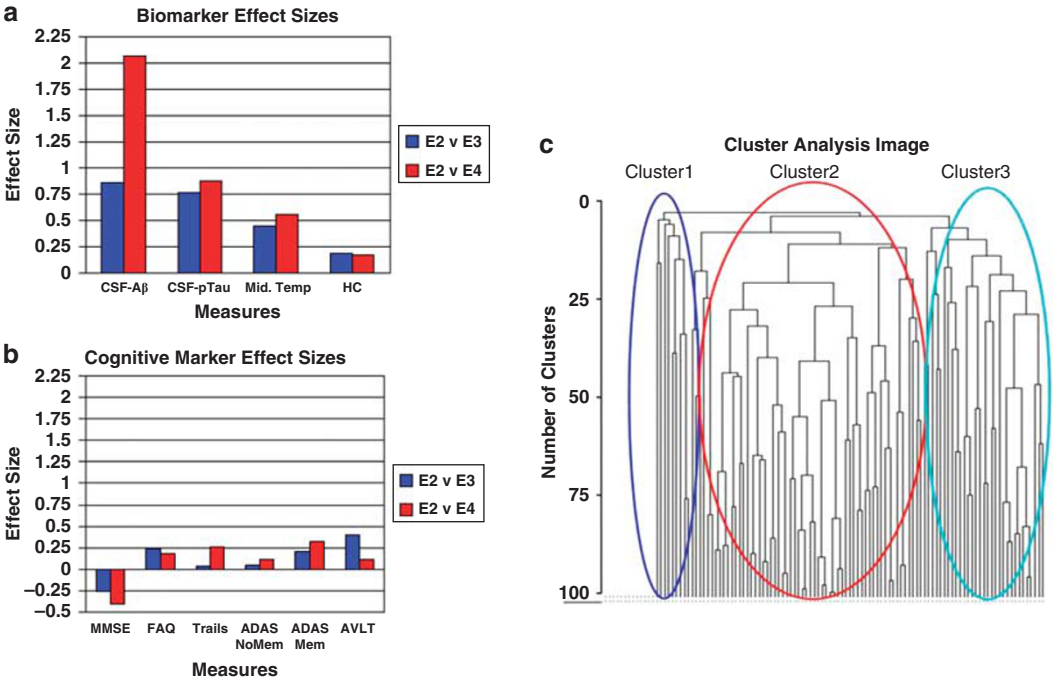

Figure 3. Biomarker profile of APOE2: differences among genotypes for CSF Ab, CSF p-tau, cognitive measures and cluster analysis. (**a**) Significant differences were present among the genotypes for CSF Ab ($P < 0.0001$). Planned contrasts indicated that the E2 group had significantly higher levels of CSF Ab than the E3 group and the E4 group. Significant differences were also present among the genotypes for CSF p-tau ($P = 0.02$). Planned contrasts indicated that the E2 group had significantly lower levels of CSF p-tau than the E3 group and the E4 group. Significant differences were present among the genotypes for middle temporal cortical thickness ($P = 0.04$). Planned contrasts indicated that the E2 group had significantly greater thicknesses than the E3 group and the E4 group. Differences among genotype groups were not present for the hippocampus ($P > 0.10$), entorhinal cortex (data not shown), and total tau (data not shown). Biomarker ESs for the contrasts described above were very large for Ab1–42 and large for p-tau. In these instances, as well as for middle temporal cortical thickness, E2 was advantaged. (**b**) Significant differences among genotype groups for cognitive measures, including MMSE, episodic memory delayed recall, and trail making speed were not present. ESs for these measures for E2-E3 and E2-E4 contrasts tended to be small, though they generally favored E2. (**c**) The cluster analysis of healthy older individuals utilized biomarker and cognitive data, but was conducted independent of APOE genotype. It yielded a robust and stable three cluster solution using Average distance methods (CCC = 1.71). The dendogram displays the iterations in the aggregation process. Cluster 2 contained the highest proportion of APOE2 cases and had a favorable biomarker profile characterized by low-CSF p-tau, high-CSF Ab1–42 and marginally better cognition (data not shown) than the other two clusters. Ab, amyloid-beta.

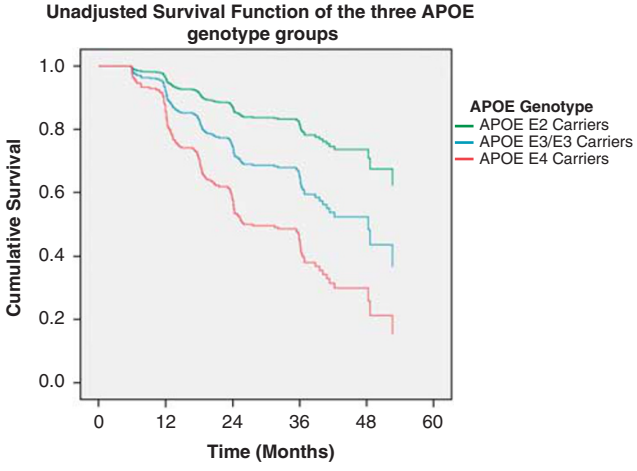

Figure 4. APOE-based Cox HR survival curves demonstrate that E2 carriers had approximately 0.50 probability of converting to AD compared to E3 cases and 0.25 probability compared to E4 cases. For the model, the global significance was $X^2 = 17.04$, $P = 0.0001$. The HR for E2 vs E3 was 2.12, $P = 0.17$; 95% confidence interval (CI) 0.66–6.81. For E2 vs E4, the HR was 3.95, $P = 0.02$; 95% CI 1.25–12.47. AD, Alzheimer's disease; HR, hazard regression.

Cox HR models to estimate risk of conversion from MCI to AD according to APOE genotype status were fitted. The survival curves of the probability for conversion to AD in each of the three APOE groups were plotted in Figure 4. In the model, APOE3 had more than 2× the hazard of conversion per unit time as compared with APOE2, while APOE4 had nearly 4× the hazard.

DISCUSSION

We conducted multiple experiments to demonstrate that molecular properties of ApoE2 have protective effects in biomarkers and neurodiagnostics relevant to AD. We discuss our main findings in turn.

First, mRNA expression levels of ApoE did not differ, but protein levels differed among ApoE isoforms, with E2 being most abundant. This in turn suggests the presence of differential post-translational modifications. There is ample evidence that while E4 is especially subject to proteolytic cleavage and degradation,[10,53] E2 may be relatively resistant to degradation as found in a conformational computational study, TR mouse studies, and now, our findings in human brain.

We found differences in the transcriptional profile in post-mortem human cortex between E2 and E3 carriers in multiple individual transcripts and pathways. The latter findings indicated

that LTP-related transcripts were downregulated, while ECM-related transcripts, including integrins, were upregulated in E2 carriers. Insofar as Ab secretion is activity dependent, reductions in LTP may attenuate release and by inference, the possibility of aggregation.[39,54] Increases in ECM-related transcripts and proteins reduce plasticity phenomena (linked to LTP) and maintain the integrity of the BBB, The 'profile' suggests reduction in activity-dependent Ab production/deposition or excitotoxicity and in the context of dendritic spine spacing and stability, relative synaptic efficiency.

Several biomarkers were modulated by APOE, with E2 carriers demonstrating a 'signature' characterized by higher levels of CSF Ab, greater middle temporal lobe cortical thickness and lower levels of p-tau in cluster analysis. Cognitive differences among genotypes were modest and nonsignificant in this cohort. This profile is consistent with a model in which E2 is specifically protective against Alzheimer's type neurodegeneration, but is not generally pro-cognitive.

E2 also had an impact at the neurodiagnostic level. It dramatically reduced the number of individuals with MCI who converted to AD. Thus, even in a group with documented memory impairment (amnestic MCI), further functional and cognitive decline was slowed or prevented by the presence of E2.

On the basis of our studies, we suggest that more abundant ApoE2 protein, in combination with its known inability to bind to the LDLR, has implications for its ability to clear Ab in an advantageous manner. Protein level may be important in driving neuroprotection, as higher levels of the ApoE2 isoform may be associated with greater cholesterol delivery to APOE receptors and perhaps, more relevantly, increase the likelihood of interactions with and subsequent clearance of Ab. In the brain, LDLRs are found on neurons and glia and account for the greater part of APOE binding, while LRP1s are highly expressed on blood vessel outer walls.[15,55] Although ApoE2's binding to LDLRs is dramatically reduced, its binding to other low-density lipid receptors (including LRP1) is unaffected. Because ApoE2 cannot transport Ab species to cellular LDLRs, where it might be endocytosed inefficiently or proven toxic, it appears likely that it differentially transports Ab to the BBB and there it binds to LRP1 receptors, thus providing more robust Ab clearance, as shown in Deane *et al*.[21]

In summary, ApoE2 may have pleiotropic effects on multiple protective signaling pathways. Evidence suggests that several fundamental molecular properties of ApoE2 isoform, including its high protein level in comparison to other isoforms as shown here and its inability to bind to LDLRs, may allow more robust interaction with Ab and/or efficient clearance of Ab through the cerebral vasculature. APOE2 carrier's transcriptional profile in cortex was characterized by reductions in expression of LTP-related molecules, perhaps reducing excitotoxic lesions or activity-dependent Ab production. Concomitant ECM/integrin-related upregulations, CypA downregulation, and increases in collagen VI and laminin protein also might reduce activity-dependent plasticity, reduce tau aggregation, and/or further maintain the integrity of the BBB. The molecular properties of ApoE2 have unusual but interpretable consequences at the neurobehavioral/neurodiagnostic level. APOE2 genotype was associated with an advantageous biomarker profile in healthy older controls, including high-CSF Ab and equivocally decreased p-tau, but not general increases in cognition. This signature likely reduces risk of AD; and as shown here, a reduced risk of MCI to AD conversion in E2 carriers. Furthermore, we have shown that high levels of the ApoE2 isoform are not disadvantageous in terms of brain function, and may be advantageous. We think that our set of observations has important implications for an E2-based drug discovery platform and understanding individual variations in cognitive aging.

CONFLICT OF INTEREST

TG has consulted for Neurocog Trials. He receives royalties for use of a cognitive test battery in clinical trials, the BACS. PD has received research support from and served as a consultant to Applied Neurosolutions. The remaining authors declare no conflict of interest.

ACKNOWLEDGMENTS

Funding for this study was provided by the Litwin Zucker Alzheimer's Disease Center (CCG, TEG, JJG, TBB, SC, PD) and GCAPP, IRP, NIMH (TMH, JEK, MH). Data collection and sharing for this project was funded by the Alzheimer's Disease Neuroimaging Initiative (ADNI) (National Institutes of Health Grant U01 AG024904). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: Abbott, AstraZeneca AB, Bayer Schering Pharma AG, Bristol-Myers Squibb, Eisai Global Clinical Development, Elan Corporation, Genentech, GE Healthcare, GlaxoSmithKline, Innogenetics, Johnson and Johnson, Eli Lilly, Medpace, Merck, Novartis AG, Pfizer, F. Hoffman-La Roche, Schering-Plough, Synarc, as well as non-profit partners like the Alzheimer's Association and Alzheimer's Drug Discovery Foundation, with participation from the US Food and Drug Administration. Private sector contributions to ADNI are facilitated by the Foundation for the National Institutes of Health (http://www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Disease Cooperative Study at the University of California, San Diego, CA, USA. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of California, Los Angeles, CA, USA. This research was also supported by NIH grants P30 AG010129, K01 AG030514, and the Dana Foundation.The ADNI investigators contributed to the design of ADNI, diagnoses, and data collection of biomarkers, but not to the analyses and writing of this report.

REFERENCES

1 Mahley RW, Huang Y. Apolipoprotein e sets the stage: response to injury triggers neuropathology. *Neuron* 2012; **76**: 871–885.
2 Mahley RW, Weisgraber KH, Huang Y. Apolipoprotein E4: a causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc Natl Acad Sci USA* 2006; **103**: 5644–5651.
3 Corder EH, Saunders AM, Risch NJ, Strittmatter WJ, Schmechel DE, Gaskell PC Jr *et al.* Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nat Genet* 1994; **7**: 180–184.
4 Bertram L, McQueen MB, Mullin K, Blacker D, Tanzi RE. Systematic meta-analyses of Alzheimer disease genetic association studies: the AlzGene database. *Nat Genet* 2007; **39**: 17–23.
5 Sudlow C, Martinez Gonzalez NA, Kim J, Clark C. Does apolipoprotein E genotype influence the risk of ischemic stroke, intracerebral hemorrhage, or subarachnoid hemorrhage? Systematic review and meta-analyses of 31 studies among 5961 cases and 17,965 controls. *Stroke* 2006; **37**: 364–370.
6 Ghebremedhin E, Schultz C, Botez G, Rub U, Sassin I, Braak E *et al.* Argyrophilic grain disease is associated with apolipoprotein E epsilon 2 allele. *Acta Neuropathol* 1998; **96**: 222–224.
7 Togo T, Cookson N, Dickson DW. Argyrophilic grain disease: neuropathology, frequency in a dementia brain bank and lack of relationship with apolipoprotein E. *Brain Pathol* 2002; **12**: 45–52.
8 Huang X, Chen PC, Poole C. APOE-[epsilon]2 allele associated with higher prevalence of sporadic Parkinson disease. *Neurology* 2004; **62**: 2198–2202.
9 Verpillat P, Camuzat A, Hannequin D, Thomas-Anterion C, Puel M, Belliard S *et al.* Apolipoprotein E gene in frontotemporal dementia: an association study and meta-analysis. *Eur J Hum Genet* 2002; **10**: 399–405.
10 Mahley RW, Huang Y, Rall SC Jr. Pathogenesis of type III hyperlipoproteinemia (dysbetalipoproteinemia). Questions, quandaries, and paradoxes. *J Lipid Res* 1999; **40**: 1933–1949.
11 Bennet AM, Di Angelantonio E, Ye Z, Wensley F, Dahlin A, Ahlbom A *et al.* Association of apolipoprotein E genotypes with lipid levels and coronary risk. *JAMA* 2007; **298**: 1300–1311.
12 Drenos F, Kirkwood TB. Selection on alleles affecting human longevity and late-life disease: the example of apolipoprotein E. *PLoS One* 2010; **5**: e10022.
13 Gerdes LU, Jeune B, Ranberg KA, Nybo H, Vaupel JW. Estimation of apolipoprotein E genotype-specific relative mortality risks from the distribution of genotypes in centenarians and middle-aged men: apolipoprotein E gene is a 'frailty gene,' not a 'longevity gene'. *Genet Epidemiol* 2000; **19**: 202–210.
14 Ward H, Mitrou PN, Bowman R, Luben R, Wareham NJ, Khaw KT *et al.* APOE genotype, lipids, and coronary heart disease risk: a prospective population study. *Arch Intern Med* 2009; **169**: 1424–1429.

15 Bu G, Apolipoprotein E. and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy. *Nat Rev Neurosci* 2009; **10**: 333–344.
16 Di Paolo G, Kim TW. Linking lipids to Alzheimer's disease: cholesterol and beyond. *Nat Rev Neurosci* 2011 May; **12**: 284–296.
17 Leduc V, Domenger D, De Beaumont L, Lalonde D, Belanger-Jasmin S, Poirier J. Function and comorbidities of apolipoprotein e in Alzheimer's disease. *Int J Alzheimers Dis* 2011; **2011**: 974361.
18 Yang DS, Smith JD, Zhou Z, Gandy SE, Martins RN. Characterization of the binding of amyloid-beta peptide to cell culture-derived native apolipoprotein E2, E3, and E4 isoforms and to isoforms from human plasma. *J Neurochem* 1997; **68**: 721–725.
19 Kim J, Basak JM, Holtzman DM. The role of apolipoprotein E in Alzheimer's disease. *Neuron* 2009; **63**: 287–303.
20 Aleshkov S, Abraham CR, Zannis VI. Interaction of nascent ApoE2, ApoE3, and ApoE4 isoforms expressed in mammalian cells with amyloid peptide beta (1-40). Relevance to Alzheimer's disease. *Biochemistry* 1997; **36**: 10571–10580.
21 Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB *et al.* apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain. *J Clin Invest* 2008; **118**: 4002–4013.
22 Jiang Q, Lee CY, Mandrekar S, Wilkinson B, Cramer P, Zelcer N *et al.* ApoE promotes the proteolytic degradation of Abeta. *Neuron* 2008; **58**: 681–693.
23 Conejero-Goldberg C, Hyde TM, Chen S, Dreses-Werringloer U, Herman MM, Kleinman JE *et al.* Molecular signatures in post-mortem brain tissue of younger individuals at high risk for Alzheimer's disease as based on APOE genotype. *Mol Psychiatry* 2011; **16**: 836–847.
24 Gomar JJ, Bobes-Bascaran MT, Conejero-Goldberg C, Davies P, Goldberg TE. Utility of combinations of biomarkers, cognitive markers, and risk factors to predict conversion from mild cognitive impairment to Alzheimer disease in patients in the Alzheimer's disease neuroimaging initiative. *Arch Gen Psychiatry* 2011; **68**: 961–969.
25 Brendza RP, Bales KR, Paul SM, Holtzman DM. Role of apoE/Abeta interactions in Alzheimer's disease: insights from transgenic mouse models. *Mol Psychiatry* 2002; **7**: 132–135.
26 Tolar M, Marques MA, Harmony JA, Crutcher KA. Neurotoxicity of the 22 kDa thrombin-cleavage fragment of apolipoprotein E and related synthetic peptides is receptor-mediated.. *J Neurosci* 1997; **17**: 5678–5686.
27 Harris FM, Brecht WJ, Xu Q, Tesseur I, Kekonius L, Wyss-Coray T *et al.* Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice. *Proc Natl Acad Sci USA* 2003; **100**: 10966–10971.
28 Soares HD, Potter WZ, Pickering E, Kuhn M, Immermann FW, Shera DM *et al.* Plasma biomarkers associated with the apolipoprotein E genotype and Alzheimer disease. *Arch Neurol* 2012; **69**: 1310–1317.
29 Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J *et al.* Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. *J Neurosci* 2008; **28**: 11445–11453.
30 Bales KR, Liu F, Wu S, Lin S, Koger D, DeLong C *et al.* Human APOE isoform-dependent effects on brain beta-amyloid levels in PDAPP transgenic mice. *J Neurosci* 2009; **29**: 6771–6779.
31 Xu PT, Schmechel D, Qiu HL, Herbstreith M, Rothrock-Christian T, Eyster M *et al.* Sialylated human apolipoprotein E (apoEs) is preferentially associated with neuron-enriched cultures from APOE transgenic mice. *Neurobiol Dis* 1999; **6**: 63–75.
32 Bell RD, Winkler EA, Singh I, Sagare AP, Deane R, Wu Z *et al.* Apolipoprotein E controls cerebrovascular integrity via cyclophilin A. *Nature* 2012; **485**: 512–516.
33 Zlokovic BV. Cerebrovascular effects of apolipoprotein E: implications for Alzheimer disease. *JAMA Neurol* 2013; **70**: 440–444.
34 Chan CS, Weeber EJ, Kurup S, Sweatt JD, Davis RL. Integrin requirement for hippocampal synaptic plasticity and spatial memory. *J Neurosci* 2003; **23**: 7107–7116.
35 Reymann KG, Frey JU. The late maintenance of hippocampal LTP: requirements, phases, 'synaptic tagging', 'late-associativity' and implications. *Neuropharmacology* 2007; **52**: 24–40.
36 Trommer BL, Shah C, Yun SH, Gamkrelidze G, Pasternak ES, Ye GL *et al.* ApoE isoform affects LTP in human targeted replacement mice. *Neuroreport* 2004; **15**: 2655–2658.
37 Buckner RL, Sepulcre J, Talukdar T, Krienen FM, Liu H, Hedden T *et al.* Cortical hubs revealed by intrinsic functional connectivity: mapping, assessment of stability, and relation to Alzheimer's disease. *J Neurosci* 2009; **29**: 1860–1873.
38 Bero AW, Yan P, Roh JH, Cirrito JR, Stewart FR, Raichle ME *et al.* Neuronal activity regulates the regional vulnerability to amyloid-beta deposition. *Nat Neurosci* 2011; **14**: 750–756.
39 Cirrito JR, Yamada KA, Finn MB, Sloviter RS, Bales KR, May PC *et al.* Synaptic activity regulates interstitial fluid amyloid-beta levels *In vivo*. *Neuron* 2005; **48**: 913–922.
40 Suberbielle E, Sanchez PE, Kravitz AV, Wang X, Ho K, Eilertson K *et al.* Physiologic brain activity causes DNA double-strand breaks in neurons, with exacerbation by amyloid-beta. *Nat Neurosci* 2013; **16**: 613–621.
41 de Vivo L, Landi S, Panniello M, Baroncelli L, Chierzi S, Mariotti L *et al.* Extracellular matrix inhibits structural and functional plasticity of dendritic spines in the adult visual cortex. *Nat Commun* 2013; **4**: 1484.
42 Wang D, Fawcett J. The perineuronal net and the control of CNS plasticity. *Cell Tissue Res* 2012; **349**: 147–160.
43 Lendvai D, Morawski M, Negyessy L, Gati G, Jager C, Baksa G *et al.* Neurochemical mapping of the human hippocampus reveals perisynaptic matrix around functional synapses in Alzheimer's disease. *Acta Neuropathol* 2013; **125**: 215–229.
44 Davis GE, Senger DR. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res* 2005; **97**: 1093–1107.
45 Morris JC, Roe CM, Xiong C, Fagan AM, Goate AM, Holtzman DM *et al.* APOE predicts amyloid-beta but not tau Alzheimer pathology in cognitively normal aging. *Ann Neurol* 2010; **67**: 122–131.
46 Chiang GC, Insel PS, Tosun D, Schuff N, Truran-Sacrey D, Raptentsetsang ST *et al.* Hippocampal atrophy rates and CSF biomarkers in elderly APOE2 normal subjects. *Neurology* 2010; **75**: 1976–1981.
47 Castellano JM, Kim J, Stewart FR, Jiang H, DeMattos RB, Patterson BW *et al.* Human apoE isoforms differentially regulate brain amyloid-beta peptide clearance. *Sci Transl Med* 2011; **3**: 89ra57.
48 Wilson RS, Bienias JL, Berry-Kravis E, Evans DA, Bennett DA. The apolipoprotein E epsilon 2 allele and decline in episodic memory. *J Neurol Neurosurg Psychiatry* 2002; **73**: 672–677.
49 Helkala EL, Koivisto K, Hanninen T, Vanhanen M, Kervinen K, Kuusisto J *et al.* The association of apolipoprotein E polymorphism with memory: a population based study. *Neurosci Lett* 1995; **191**: 141–144.
50 Deary IJ, Whiteman MC, Pattie A, Starr JM, Hayward C, Wright AF *et al.* Apolipoprotein e gene variability and cognitive functions at age 79: a follow-up of the Scottish mental survey of 1932. *Psychol Aging* 2004; **19**: 367–371.
51 Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW *et al.* Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol* 2010; **9**: 119–128.
52 Shaw LM, Vanderstichele H, Knapik-Czajka M, Clark CM, Aisen PS, Petersen RC *et al.* Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects. *Ann Neurol* 2009; **65**: 403–413.
53 Jones PB, Adams KW, Rozkalne A, Spires-Jones TL, Hshieh TT, Hashimoto T *et al.* Apolipoprotein E: isoform specific differences in tertiary structure and interaction with amyloid-beta in human Alzheimer brain. *PLoS One* 2011; **6**: e14586.
54 Palop JJ, Mucke L. Synaptic depression and aberrant excitatory network activity in Alzheimer's disease: two faces of the same coin? *Neuromolecular Med* 2010; **12**: 48–55.
55 Holtzman DM, Herz J, Bu G. Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease. *Cold Spring Harb Perspect Med* 2012; **2**: a006312.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)