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A SAGE study of apolipoprotein E3/3, E3/4 and E4/4 allele-specific gene expression in hippocampus in Alzheimer disease

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Abstract

APOE4 allele is a major risk factor for late-onset Alzheimer disease (AD). The mechanism of action of *APOE* in AD remains unclear. To study the effects of *APOE* alleles on gene expression in AD, we have analyzed the gene transcription patterns of human hippocampus from *APOE3/3*, *APOE3/4*, *APOE4/4* AD patients and normal control using Serial Analysis of Gene Expression (SAGE). Using SAGE, we found gene expression patterns in hippocampus of *APOE3/4* and *APOE4/4* AD patients differ substantially from those of *APOE3/3* AD patients. *APOE3/4* and *APOE4/4* allele expression may activate similar genes or gene pools with associated functions. *APOE4* AD alleles activate multiple tumor suppressors, tumor inducers and negative regulator of cell growth or repressors that may lead to increased cell arrest, senescence and apoptosis. In contrast, there is decreased expression of large clusters of genes associated with synaptic plasticity, synaptic vesicle docking and fusing and axonal/neuronal outgrowth. In addition, reduction of neurotransmitter receptors and Ca⁺⁺ homeostasis, disruption of multiple signal transduction pathways, loss of cell protection, and perhaps most notably, mitochondrial oxidative phosphorylation/energy metabolism are associated with *APOE3/4* and *APOE4/4* AD alleles. These findings may help define the mechanisms that *APOE4* contribute increased risk for AD and identify new candidate genes conferring susceptibility to AD.

Keywords

Apolipoprotein E; Alzheimer disease; Serial Analysis of Gene Expression (SAGE); apoptosis; signal pathways

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The AD SAGE tag count and lists were submitted to NCBI Gene Expression Omnibus (GEO). The GEO accession number is GSE6677.

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Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized by progressive deterioration of memory and cognition with specific neuropathological changes, including extracellular amyloid –containing plaques, intracellular neurofibrillary tangles (NFT) of abnormally phosphorylated tau protein, and degeneration of the cholinergic neurons of the basal forebrain (Auld et al., 2002). A relatively small number of early onset AD families exhibit genetic linkage to mutations in presenilin-1 (PS1), presenilin-2 (PS2), and amyloid precursor protein (APP) genes (George-Hyslop et al., 2000;Tamouza et al., 2000;Holmes et al., 2000). The three major isoforms of human apolipoprotein E (apoE2, E3 and E4) are associated with both risk and age of onset of AD and response to brain injury. The *APOE4* allele is associated with increased risk and earlier age of onset; while the *APOE2* allele decreases risk and delays AD onset in population studies compared to the *APOE3* allele. (Corder et al., 1993;Saunders et al., 1993;Poirier et al., 1993;Rebeck et al., 1993). The *APOE4* allele increases risk and lowers the age of onset of AD in a dose-dependent manner (Corder et al., 1994). In addition, the *APOE4* allele has been implicated in poorer neurological recovery from head injury, cerebral hemorrhage, and cognitive status after cardiac bypass surgery (Alberts et al., 1995;Tardiff et al., 1997). The exact role that apoE plays in neuronal metabolism and function is, at present, poorly understood.

Several microarray studies involving human AD brain have been published (Eikelboom et al., 2000;Auld et al., 2002;Mufson et al., 2002;Colangelo et al., 2002;Beckmann et al., 2001;Pasinetti and Ho, 2001;Blalock et al., 2004). These studies have yielded important new insights regarding changes in gene transcription in AD brain. This laboratory recently extended microarray studies by comparing *APOE3/3* and *APOE4/4* allele-specific microarray gene expression profiles from hippocampus of AD cases (Xu et al., 2006).

Serial analysis of gene expression (SAGE) is a sequencing-based technique used to quantify the relative expression levels of thousands of transcripts by sequencing concatemers of short sequence tags (10 bp +restriction site) derived from biological samples (Velculescu et al., 1995). This technique has several important advantages compared to other techniques for detection of tissue specific expression (Evans et al., 2002). First, SAGE creates a permanent, quantitative record of the set of sequences transcribed in a given tissue or cell population making it suited for the quantitative analysis of gene transcripts and detection of new and/or novel genes associated with diseases, such as AD. Second, SAGE can detect small changes in expression levels (Scott and Chrast, 2001). Third, transcripts that are over or under-expressed can be detected equally well (Iyer and Struhl, 1996). The SAGE quantification profiling of AD based on *APOE* genotypes (alleles) has not been documented in previous studies.

We now extend our gene transcription studies by generating and analyzing *APOE* allele specific human SAGE libraries derived from hippocampus from AD patients. The hippocampus is heavily involved in the neuropathology of AD and is often selected for both neuropathological and molecular analysis (Markesbery, 1997). In this study, we selected hippocampus samples from AD cases with *APOE4/4*, *APOE3/4*, and *APOE3/3* genotypes and controls of *APOE 3/3* genotype.

Results

Characterization of SAGE libraries

We generated four SAGE libraries using human brain hippocampus RNA isolated from AD patients carrying *APOE3/3*, *APOE3/4* and *APOE4/4* alleles, and a normal control (*APOE3/3*). A total of 288,661 tags, with an average of 72,165 tags for each library, were

extracted from sequence files from these four SAGE libraries using e-SAGE software (Table 2). These tags correspond to 95,096 (32.9 %) tags of different transcripts, with an average of 23,774 unique tags per library. These unique tags are mapped to 54,292 unique UniGene clusters. Comparison of *APOE* allele-specific AD SAGE libraries with control library and between AD SAGE libraries indicated remarkable similarity in expression patterns or profiles (Figure 1). The correlation coefficients were in the range of 0.89 to 0.99. The eSAGE database comparisons revealed similar numbers of total tags compared between each pair (Table 3, Fig. 2A). In *APOE3/4* AD and *APOE4/4* AD compared with both *APOE3/3* AD and control, larger number of tags (625-918) showed changes greater than two-fold ($P < 0.05$) than tags (156) in the *APOE3/3* AD vs. control (Table 3, Fig. 2B). Similarly, there are more up- and down-regulated total mapped UniGene clusters (Fig 2C) and unique UniGene clusters (Fig. 2D) in *APOE3/4* AD and *APOE4/4* AD vs. *APOE3/3* AD and *APOE3/3* control than those in *APOE3/3* AD vs. control. Less than one quarter (18.8-21.2 %) of the tags did not map to any UniGene cluster (orphan tags) (Table 2). These results are consistent with previously reported SAGE libraries (Gunnarsen et al., 2002; Scott and Chrast, 2001; Anisimov et al., 2002).

Gene expression in hippocampus of *APOE3/3*, *APOE3/4* and *APOE4/4* AD compared with control

To explore whether specific *APOE* alleles may regulate or affect different genes in AD, we compared differentially expressed genes in *APOE3/3* AD, *APOE3/4* AD and *APOE4/4* AD versus *APOE3/3* normal controls. Comparing *APOE3/3* AD and *APOE3/3* control SAGE libraries, the distribution of SAGE tag counts are quite similar with most of the SAGE tags lying close to the 45-degree identity line (Figure 1A). A total of 37,900 tags were compared and 156 tags (0.41 %) were found with significant tag count differences ($P < 0.05$). One hundred tags could be specifically assigned to unique UniGene Clusters (Table 3). Of this total, forty-eight gene transcripts are up-regulated and 52 genes down-regulated (Table 3, Fig. 2D). For *APOE3/4* AD and *APOE4/4* AD vs. control, 39,059 and 36,632 SAGE tags were compared and 906 tags (2.32 %) and 625 tags (1.71 %) showed significant results, respectively. 523 and 320 tags were assigned to unique UniGene Clusters and 97 and 108 UniGene clusters are up-regulated and 426 and 212 UniGene clusters down-regulated, respectively (Fig. 1B,1C, Table 3, Fig. 2D). Direct comparison between the three AD SAGE libraries and control reveal *APOE* allele-specific quantitative differences. In general more total gene transcripts are up-regulated and down-regulated in both *APOE3/4* AD and *APOE4/4* AD than in *APOE3/3* AD hippocampus relative to control (Table 3; Fig. 1, 2).

Among the upregulated genes in *APOE4/4* AD and *APOE3/4* AD versus control, 15 out of 108 (13.9 %) and 97 (15.5 %) up-regulated transcripts overlapped (Fig 5A). Only 4 genes (3.7 %) in *APOE4/4* AD vs. control and 3 genes (3.1 %) in *APOE3/4* AD vs. control shared upregulated genes in *APOE3/3* AD vs. control (Fig. 5A). Among the 212 downregulated gene transcripts in *APOE4/4* AD versus control, 135 genes (63.7 %) were also found downregulated in 426 genes in the comparison of *APOE3/4* AD vs. control (Fig 5B). In contrast, only 16 genes (7.5 %) in the *APOE4/4* AD vs. control and 28 genes (6.6 %) in the *APOE3/4* AD vs. control were shared downregulated genes in *APOE3/3* AD vs. control (Fig. 5B). Detail overlapped genes and fold changes were listed in Supplementary Table 1-3 online. These findings support the possibility that *APOE4* gene expression may regulate different genes or gene pools from the *APOE3* gene expression in hippocampus of AD patients.

Gene expression of *APOE4/4* AD and *APOE3/4* AD hippocampus compared with *APOE3/3* AD

A total of 918 and 771 SAGE tags are significantly ($p < 0.05$) differentially expressed in *APOE3/4* AD vs. *APOE3/3* AD and *APOE4/4* AD vs. *APOE3/3* AD, respectively. These tags were specifically assigned to 499 and 428 unique UniGene clusters with 131 and 172 gene transcripts up-regulated and 368 and 256 genes down-regulated in these two comparisons (Table 3, Fig. 2D). Among up-regulated gene transcripts, 20 genes out of 131 transcripts (15.3 %) in *APOE3/4* AD vs. *APOE3/3* AD and 172 (11.6 %) genes in *APOE4/4* AD vs. *APOE3/3* AD are shared up-regulated genes in these two comparisons (Fig. 6A, Supplementary table 4). In contrast, 167 out of 368 (45 %) and 256 (65 %) are shared down-regulated genes in these two comparisons (Fig. 6B). These observations suggest that *APOE4* allele gene expression in hippocampus of AD may down-regulate or inactivate a subset of common genes and/or the same gene pools with similar or associated functions.

Among the upregulated genes in the hippocampus of *APOE3/4* and *APOE4/4* AD compared to *APOE3/3* AD and *APOE3/3* control, 66 and 58 genes shared upregulation in both comparisons (Fig. 7A, Supplementary Table 7, 8). Among the down-regulated genes in the hippocampus of *APOE3/4* AD and *APOE4/4* AD compared to *APOE3/3* AD and *APOE3/3* control, 219 and 120 downregulated gene transcripts overlapped (Fig. 7B, Supplementary Table 7, 8).

Gene Ontology: Quantitative differences of differentially expressed genes in *APOE3/3*, *APOE3/4* and *APOE4/4* ADs compared to control

Molecular functional categorization analysis (EASE) demonstrates that transcripts of protein binding and transporter, receptor binding and signaling, nucleotide binding, transferase and kinase activity are among the most elevated gene categories in both *APOE3/4* AD vs. control and *APOE4/4* AD vs. control compared to *APOE3/3* AD vs. control (Fig. 3A, Table 4). In *APOE3/4* AD vs. control genes involved in protein binding and transporter, receptor binding and signaling, carriers and enzyme activity are the most up-regulated transcripts relative to both *APOE3/3* and *APOE4/4* AD compared with control (Fig. 3A Table 4). In *APOE4/4* AD hippocampus, larger numbers of transcripts encoding hydrolase, electron/channel transporter, kinase and oxidoreductase are up-regulated compared to *APOE3/3* AD and *APOE3/4* AD.

A large number of gene transcripts are down-regulated in *APOE3/4* and *APOE4/4* AD relative to *APOE3/3* AD when compared to control in 14 selected functional categories (Fig. 3B, Table 4). The *APOE3/4* AD SAGE library has the most transcripts down-regulated in 13 of the 14 selected functional categories of genes. These findings are consistent with recent published microarray gene expression studies (Xu et al., 2006) that *APOE4* gene expression in hippocampus may activate or inhibit multiple categories of protein function and/or protein expression compared with *APOE3* expression in different AD patients.

Gene Ontology: Quantitative differences of differentially expressed genes in *APOE* allele-specific ADs

To investigate whether there are fundamental differences in gene expression in AD patients carrying one, two, or no copies of the *APOE4* allele the molecular functional categories of gene transcripts between *APOE3/4* vs. *APOE3/3* and *APOE4/4* vs. *APOE3/3* AD SAGE libraries were compared (Fig. 4, Table 5). Three functional categories of transcripts (hydrolase, nucleic acid binding and transferase) have more upregulated transcripts in *APOE4/4* AD than *APOE3/4* AD hippocampus (Fig. 4A). Several functional categories have larger numbers of elevated transcripts in *APOE4/4* AD vs. *APOE3/4* AD than in *APOE4/4* AD vs. *APOE3/3* AD and *APOE3/4* AD vs. *APOE3/3* AD. Categories include protein

binding and transport, receptor binding and signaling, nucleotide binding, metal ion binding, hydrolase and kinase (Fig. 4A). In contrast, there are fewer significant down-regulated gene transcripts in all 14 selected functional categories identified in the comparison of *APOE4/4* AD vs. *APOE3/4* AD than in the comparisons of *APOE4/4* AD vs. *APOE3/3* AD and *APOE3/4* AD vs. *APOE3/3* AD (Fig. 4B, Table 5).

Functional clusters of 5-fold up- and down-regulated genes in *APOE* allele-specific AD compared with control

To examine genes whose transcription is substantially altered we chose five fold transcription alteration as the cutoff and directly compared major functional categories that are 5-fold or greater up- or down-regulated genes in *APOE3/3*, *APOE3/4*, *APOE4/4* AD tissues compared to *APOE3/3* control (Tables 7-11; Supplementary Table 9-11 online). One striking difference is that *APOE3/4* and *APOE4/4* alleles strongly up-regulate gene transcription of tumor suppressor, cell arrest and negative regulators of cell growth and repressors (Table 7). Other clear differences are up-regulation of genes associated with protein modification and oxidoreductase. Several gene clusters are up-regulated in all three AD genotypes, including calcium signaling and homeostasis, cell adhesion, myelinogenesis, transcription factors and regulators, cell growth and differentiation (Table 7).

Functional clusters containing the greatest number of 5-fold down-regulated transcripts in *APOE3/4* AD are genes involved in regulating signal transduction pathways (including G-protein mediated signaling), synaptic plasticity and formation, synaptic vesicle docking and fusion, and axonal guidance, neuronal outgrowth and neurogenesis (Table 9, 10). Although individual calcium signaling transcripts are up-regulated (Table 7) in all three *APOE* genotypes of ADs vs. control, signal transduction pathways in general are down-regulated especially in *APOE3/4* AD and *APOE4/4* AD compared with control (Table 10, Supplementary Table 10, 11).

Other groups of down-regulated gene transcripts include proteins that regulate cell growth, differentiation and development, and protein biosynthesis. Apoptosis inducers are down-regulated especially in *APOE3/4* AD hippocampus compared with *APOE3/3* control (Supplementary Table 10). Negative regulators or repressors and genes involved in negative growth regulation are down-regulated in *APOE4/4* AD and *APOE3/4* AD compared to control (Supplementary Table 10, 11). There are more down-regulated gene transcripts in *APOE3/4* AD than in *APOE4/4* AD that involves protein modification, ubiquitination, ER to Golgi transport and vesicle trafficking and RNA splicing and RNA editing have (Supplementary Table 9-11). Consistent with previously reported results, multiple gene transcripts encoding neurotransmitter receptors, energy metabolism and mitochondrial oxidative phosphorylation and cytoskeleton and structure proteins are significantly down-regulated in both *APOE3/4* AD and *APOE4/4* AD hippocampus relative to *APOE3/3* control (Table 11, Supplementary Table 9-11).

Functional clusters of 5-fold up- and down-regulated genes in *APOE3/4* and *APOE4/4* AD compared with *APOE3/3* AD

Comparing *APOE3/4* and *APOE4/4* AD with *APOE3/3* AD and control, there are similar patterns of the functional clustering of highly up-regulated (Table 7, 8) and down-regulated genes (Table 9-11; Supplementary Table 12, 13). Transcripts associated with signaling transduction are among the most elevated category in the both *APOE3/4* AD and *APOE4/4* AD (Table 8). Tumor suppressor and cell arrest gene transcripts are up-regulated in both *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* AD (Table 8), as well as *APOE3/3* control (Table 7). In *APOE3/4* AD cases compared with *APOE3/3* AD, there are more up-regulated transcripts encoding cell adhesion and myelinogenesis, apoptosis, ER to

Golgi transport and vesicle trafficking, and stress response and chaperone (Table 8, Supplementary Table 12, 13). In the *APOE4/4* AD vs. *APOE3/3* AD, there are more upregulated transcripts in the functional categories of cell growth and differentiation, protein modification, RNA binding and editing.

In *APOE3/4* AD compared with *APOE3/3* AD, signal transduction, calcium signaling and homeostasis, synaptic plasticity, synaptic vesicle docking and fusing, neurite outgrowth, RNA processing and editing, cell adhesion and myelinogenesis are the most down-regulated transcript clusters (Supplementary Table 12). In the functional categories of negative regulators of cell growth and repressors, protein modification and ubiquitin metabolism, more gene transcripts are down-regulated in *APOE4/4* AD vs. *APOE3/3* AD than in *APOE3/4* AD vs. *APOE3/3* AD (Supplementary Table 12, 13). Looking at gene expression one obvious difference is the striking number of mitochondrial oxidative phosphorylation and complex I subunits that are down-regulated in *APOE3/4* and *APOE4/4* AD relative to both *APOE3/3* AD and control. Additionally, stress response, chaperone and detoxification, and ER-Golgi transport and cytoskeletal and structural genes are also down-regulated in both *APOE3/4* and *APOE4/4* AD compared with *APOE3/3* AD and *APOE3/3* control (Table 11; Supplementary Table 12, 13).

Discussion

SAGE libraries and data are judged to be of high quality by four criteria. First, there is no GC content bias in all four libraries (Margulies and Innis, 2000). Second, the frequency of both the duplicated ditags and linker contamination is less than 0.5 %, equivalent or below that of published SAGE libraries (Velculescu et al., 1995). Third, comparisons between SAGE libraries demonstrate that the correlation coefficients are in the range of 0.89 to 0.99 (Figure 1) and only 0.41 % to 2.32 % of the tags show changes greater than two-fold (p -value < 0.05) (Table 3). Representative regulated genes were verified by real-time PCR (Table 6) on individual hippocampal RNA samples with the same *APOE* genotype. Lastly, in separate experiments our SAGE libraries tag count further confirm the qualitative changes in gene expression profiles previously observed in *APOE4/4* and *APOE3/3* allele-specific AD microarray experiments (Xu et al., 2006).

SAGE data demonstrates, as might be expected, that the hippocampal SAGE patterns in *APOE 3/4* AD are similar to those observed in the *APOE 4/4* AD. SAGE data indicate that *APOE4* gene expression in AD hippocampus induces or significantly up-regulates more than two times and inactivates or down-regulates more than four times the number of genes when compared to *APOE3* expression in *APOE3/3* control and *APOE3/3* AD (Table 3, Fig. 2). EASE molecular functional categorization demonstrates several categories have more elevated genes and all categories have more down-regulated genes in both *APOE3/4* AD and *APOE4/4* AD vs. *APOE3/3* control than in *APOE3/4* AD vs. *APOE3/3* control (Fig. 3, Table 4). Larger percentages of gene transcripts share up- and down-regulation in hippocampus in both *APOE3/4* AD and *APOE4/4* AD vs. *APOE3/3* control and *APOE3/3* AD than in *APOE3/3* AD vs. *APOE3/3* control (Fig. 5, 6).

Activation of tumor suppressor and arrest, and negative regulators of cell growth and repressors are associated with *APOE3/4* AD and *APOE4/4* AD

One striking example of significantly up-regulated gene transcripts include tumor suppressors, tumor inducers or tumor associated proteins, and negative regulators of transcription or repressors in both *APOE3/4* AD and *APOE4/4* AD hippocampus compared with control (Table 7) and *APOE3/3* AD (Table 8). Activation and overexpression of these genes (MCC, SMAD2, IGFBP7, DLC1, HIPK2 and MEG3) may increase cell cycle arrest in senescent and/or apoptotic cells, be associated with malfunction of DNA repair and

modification (MALAT1, TPM3/TC22, XIST, MUTYH, EMP3, ELAC2 and ECRG4), and inhibit or decrease cell growth and gene transcription (TRIM33/TIF1G, ZHX3, PCOLN3, and DMAP1). Activation of tumor suppressor and oncogenesis genes in *APOE3/4* AD and *APOE4/4* AD may increase cell cycle arrest and inhibit cell differentiation and cell cycle reentry of neurons in AD brain. Previous studies have found that the relations between tumor suppressors and AD largely involve apoptotic pathways (e.g. p53) (Galderisi et al., 2001). Up-regulation of tumor suppressor related transcription factors is previously reported in incipient AD (Blalock, 2004). Details of gene functions and references in discussion section are contained in Supplementary Discussion online.

Global defects of synaptic plasticity, LTP and memory in *APOE3/4* and *APOE4/4* AD

A large cluster of gene transcripts involved in synaptic plasticity and synapse formation are significantly down-regulated in both *APOE3/4* AD and *APOE4/4* AD hippocampus (Table 9; Supplementary table 9-11) (Supplementary Table 12, 13). These results strongly suggest that *APOE4* gene expression in the hippocampus of AD patients may inactivate or disrupt gene transcription involved in synaptic plasticity, long term potentiation and depression (EFNB3, EPHA4, SNAP91 and PPP3CA), inhibit or decrease neuronal outgrowth, synaptic regeneration, remodeling and formation of new connections (GAP43, BASP1, NPTX1, NPTXR, SYNGR1, SYNGR3 and SYNPO), reduce synapse stability, and disturb the functions or activities of ion channel and neuronal signaling (PSD95, DLGAP1/GKAP and NMDAR). These data suggest *APOE4* expression may likely directly or indirectly inhibit or disrupt neuronal outgrowth, synaptic regeneration, remodeling and formation new connection in the hippocampus of *APOE3/4* AD and *APOE4/4* AD patients.

Presynaptic vesicle docking and fusion are disrupted in *APOE3/4* AD and *APOE4/4* AD

SAGE data reveal that multiple active zone proteins involving in presynaptic vesicle docking and fusion are significantly down-regulated in both *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* AD (Table 9; Supplementary table 10,11) as well as *APOE3/3* control (Supplementary Table 12, 13). Presynaptic terminals are organized into junctions known as active zones where the vesicular protein synaptotagmin (e.g., SYT1) serves as the main Ca^{2+} sensor for neurotransmitter release (Augustine, 2001; Koh and Bellen, 2003). The highly specialized uptake and exocytosis system of synaptic vesicle traffic has been studied extensively as a model of membrane fusion. The fusion reaction begins with the assembly of syntaxin, SNAP25 and synaptobrevin (Singh et al., 2004) into a core complex that serves as a SNAP receptor, or SNARE. These down-regulated gene transcripts play the critical roles in regulating synaptic membrane exocytosis (RIMS2 and RIMS3), serving as calcium sensors (SYT1 and SYT13), forming both SNARE complex (VAMP2 and NAPB) and synaptic vesicle (SV2A and VAPB), and scaffolding presynaptic active zone assembly or functions (RIMBP, DNM1, CPLX1 and PPFIA4/Liprin) (Augustine, 2001; Koh and Bellen, 2003).

These losses of genes encoding proteins functionally related to synaptic vesicle docking and fusing and synaptic plasticity appear to be *APOE4* allele(s) specific and are found only in *APOE3/4* AD and *APOE4/4* AD (Table 9). In AD loss of synapses correlates with cognitive decline (DeKosky and Scheff, 1990; Terry et al., 1991). Reduction of synaptic vesicle trafficking related genes including synaptophysin, synapsin, synaptotagmin, AP180, DNM1 and RAB3A have been reported previously in AD (Eikelboom et al., 2000; Ho et al., 2001; Bouhouche et al., 1999; Yao et al., 2003). A loss of afferents from entorhinal cortex are believed to underlie the synapse loss seen in early AD (Scheff et al., 2005). Coleman and Yao (Coleman and Yao, 2003) provide evidence that in addition to loss of synapses, AD synapses may not function effectively or appropriately by decreasing transcripts related to synaptic vesicle trafficking (Yao et al., 2003). These findings suggest that losses of multiple

presynaptic active zone proteins in AD with *APOE4* allele(s) may directly or indirectly disrupt normal synaptic vesicle docking and fusing and lead to disturbance of neuronal circuitry and activities and consequently result in cognitive impairment.

Downregulation of genes promoting axonal guidance, neuronal outgrowth and neurogenesis in *APOE3/4* and *APOE4/4* AD

Functional clusters of gene transcripts involved in axonal guidance, neurite outgrowth and neurogenesis are significantly down-regulated (or inhibited) in both *APOE3/4* AD and *APOE4/4* AD hippocampus compared with *APOE3/3* control (Table 9) and AD hippocampus (Supplementary Table 12, 13). These downregulated gene transcripts are involved in regulating neuronal axon guidance, dendritic spine morphogenesis and growth cones (SLIT1, DOK-6, NGEF, PPFIA4 and CDH10); modulating neurite outgrowth, neuronal polarity and neuronal development (THY1, GSK3B, GAS7, PPP1R9B, AF1q, TM4SF2, RUSC1 and DULLARD); and mediating neurogenesis, axonal regeneration and plasticity and neurodegeneration (RTN4R, ELAVL3, ITM2B, ITM2C and TPP1). These findings are significant in that they may provide some insight into previous observations that *APOE4* protein decreases neurite outgrowth and neuronal branching compared with *APOE3* (Nathan et al., 1994).

Changes in calcium signaling and homeostasis and neurotransmitter receptors and regulatory proteins

A large cluster of gene transcripts modulating calcium signaling homeostasis and encoding both excitatory (AMPA and NMDA) and inhibitory (GABA-A and GABA-B) neurotransmitter receptors are significantly down-regulated in *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* control (Table 10, 11) and *APOE3/3* AD (Supplementary Table 12, 13). The gene transcription of CAMK2A, CAMK2D, CALM2 and CALM3 are also down-regulated in *APOE4/4* ADs compared with *APOE3/3* ADs in microarray analysis (Xu et al., 2006). Calcium signaling is crucial for several aspects of plasticity at glutamatergic synapses and in the postsynaptic density (PSD). Intracellular calcium is one of the most extensively disrupted transduction systems in AD (Prolla and Mattson, 2001; Stutzmann, 2005). Excess glutamate causes neurotoxicity and may contribute to cellular damage in neurological disorders such as stroke, trauma (Harada and Sugimoto, 1998; Su et al., 2003), Alzheimer disease (AD), amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1995), and Huntington disease (Choi, 1988; Kanai et al., 1993). The loss and/or down-regulation of calcium signaling and both excitatory and inhibitory neurotransmitter receptors in the hippocampus of AD patients carrying *APOE4* allele(s) could potentially affect or decrease LTP, learning and memory as well as increase neuroexcitotoxicity, apoptosis and neurodegeneration.

Multiple signal transduction gene pathways are significantly downregulated in hippocampus of *APOE3/4* AD and *APOE4/4* AD

The functional cluster of signal transduction pathways contains the most down-regulated gene transcripts in *APOE3/4* and *APOE4/4* AD compared with *APOE3/3* AD (Supplementary Table 12, 13) and *APOE3/3* control (Table 10). These signaling molecules regulate a variety of signal transduction pathways and cascades including mitogen-activated protein (MAP) kinase cascades (MAPK9, MAPK8IP2 and MAP2K1), NF- κ B signaling cascades (BTKR, T1/NIBP, COMMD7), protein kinases (PRKACB, PRKAR1B, CSNK2B, ILK, NLK, ARAF1), protein tyrosine phosphatases (PTPN5, PPFIA4 and PTPRN), and G-protein signaling (GNG3, GNG, GNB5, GNAO, GNAZ, GPR51/GABBR2, RGS4 and RGS6). Several proteins may mediate regulatory signal transduction pathways in neurons to modify neuronal cytoarchitecture (TAGLN3/hNP22) and promote actin repolymerization and/or cytoskeletal reorganization (CHN1, CDC42BPA and CAP).

Down-regulation of chaperone, stress response and detoxification gene transcription is associated with *APOE3/4* AD and *APOE4/4* AD

Several gene transcripts encoding proteins that protect cells and neurons from heat shock and oxidative stresses and detoxification are downregulated in hippocampus of *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* AD and control (Table 11, Supplementary Table 12, 13). These proteins function as chaperones in the cellular heat shock response to remove denatured or incorrectly fold proteins (NAJA4, DNAJC8, STCH, HSPCB and AHSA2), detoxify hydrogen peroxide to protect against oxidative damage or stress (OXR1 and GPX1), suppress toxicity by sequestering poly (Q) and/or its aggregation (MLF2 and ASNA1), and promote clearance of apoptotic cells (C1QA). It is essential for cells to control the production and activity of such molecules as reactive oxygen species (ROS) in order to protect the genome and regulate cellular processes such as stress response and apoptosis (Elliott and Volkert, 2004).

Global defects of multiple mitochondrial oxidative phosphorylation and energy metabolism in *APOE3/4* and *APOE4/4* AD

In AD pathogenesis and other neurodegenerative disorders, increasing evidence implicates mitochondrial dysfunction and defects in oxidative phosphorylation (OXPHOS), leading to oxidative damage and defects in energy metabolism manifested by down-regulation in mitochondrial number (Ojaimi and Byrne, 2001; Manczak et al., 2004). A striking number of mitochondrial oxidative phosphorylation and complex I subunits (e.g. cytochrome c oxidases, NADH dehydrogenases, ATP synthases, etc.) are significantly down-regulated in *APOE3/4* AD and *APOE4/4* AD patients compared with *APOE3/3* AD (Supplementary Table 12, 13) and *APOE3/3* controls (Table 12). These SAGE data are consistent with recent published microarray results (Xu et al., 2006). COX defects have been suggested as a primary etiologic event (Davis and Weeks, 1997) and this study suggests that in *APOE3/4* AD and *APOE4/4* AD compared to *APOE3/3* AD the greater global defects of mitochondrial energy metabolism and mitochondrial/oxidative damage may contribute to increased risk.

In summary, SAGE data, taken in conjunction with recent results on *APOE* allele-specific expression from multiple patients using microarrays (Xu et al., 2006) together, suggest that gene expression in the hippocampus of AD *APOE3/4* and *4/4* patients are different from that observed in *APOE 3/3* AD patients and offers support for the hypothesis that *APOE3/4* and *APOE4/4* gene expression are related and may regulate similar gene pools with associated functions compared to the *APOE3* gene expression.

Experimental methods

Human brain samples and pathological assessment

Human brain tissues were collected in the Kathleen Price Bryan Brain Bank, in the Duke University Alzheimer Disease Research Center, and the Center for Human Genetics' Brain Bank, Duke University Medical Center, following the rapid autopsy protocol (Hulette et al., 1997). The hippocampus was dissected at the time of autopsy and matching 100-200 mg portions of CA 1-4 was removed and used for RNA isolation and expression studies. We chose AD patients of apolipoprotein E4/4 (*APOE4/4*), *APOE3/4*, and *APOE3/3* genotypes and controls with the *APOE 3/3* genotypes judged cognitively and pathologically normal. Clinical information on all patients was collected from clinic and hospital records. The pathological diagnosis of AD was established according to CERAD criteria (Mirra et al., 1991) and the degree of AD pathological changes was staged according to Braak (Braak and Braak, 1991). We analyzed selected AD patients with pathological changes at the Braak and Braak stage IV or V (B&B stage IV or V) and compared them with cognitively and

pathologically normal controls (B&B stage I) (Table 1). We chose hippocampal samples with mostly short post-mortem delay. RNA was analyzed using gel electrophoresis, bioanalyzer analysis, and judged by microarray analysis to have excellent 5' to 3' ratio.

Construction of human SAGE libraries

Total RNA was isolated from frozen hippocampal samples of AD patients and controls using TRIzol reagent (Invitrogen, Carlsbad, CA) as previously described (Xu et al., 2006). For SAGE library construction, we used standard protocols as described by Velculescu et al (1995) with minor modifications. Briefly, SAGE was performed with 10 µg total RNA isolated from human brain hippocampus samples as outlined above. The cDNA was prepared using the SuperscriptII cDNA synthesis kit (Invitrogen) with gel-purified 5'-biotinylated Oligo(dT)₁₈ (Integrated DNA Technologies, Coralville, IA), according to the manufacturer's protocol. *Nla*III and *Bsm*FI restriction enzymes (New England Biolab, Beverly, MA) were used for tag generation. *Bsm*FI digestion was performed at 37°C for 2.5 h (instead of 65 °C) using 40 units *Bsm*FI in a 300 µl reaction volume with supplied buffer. After a 3-hour concatemerization step, concatemers were heated at 65 °C for 10 minutes, following by 2 minutes on ice to enhance cloning efficiency. Purified concatemers were subsequently cloned in the *Sph*I site of pZero-1 (Invitrogen) and transformed in competent ElectroMax DH10B cells (Invitrogen) using a 0.1 cm cuvette and the Gene Pulser II (BioRad). Individual SAGE library clones were selected and PCR amplified using 96-well format Qiagen Real minipreps, and sequenced with ABI 3700 capillary sequencer using BigDye chemistry.

SAGE tag extract and statistical analysis

SAGE tags (10 bp) were extracted from the PHD files with eSAGE software using a threshold value of PHRED 20 for each base (Margulies and Innis, 2000). The SAGE tags were extracted from individual sequence files from each SAGE library (Table 2). The SAGE tag databases were compared with each other, such as AD vs. control and *APOE4/4* AD vs. *APOE3/3* AD SAGE tag databases, etc. using eSAGE software (Table 3). The compared SAGE databases were mapped to UniGene Homo Sapiens tag-to-gene mapping set (build 182, www.ncbi.nlm.nih.gov/UniGene).

The Chi-square test and Fisher exact test were employed to test the difference in tag counts between two samples as previously described (Man et al., 2000). For each particular Tag A, we arranged the data in a 2×2 table.

	Library 1	Library 2	
Tag A	n_{11}	n_{12}	$N_{1.}$
Others	n_{21}	n_{22}	$N_{2.}$
	$N_{.1}$	$N_{.2}$	$N.$

The tag counts for Tag A in sample 1 and sample 2 are designated as n_{11} and n_{12} , respectively. The n_{21} and n_{22} are the sum of the remaining tags in library 1 and library 2, respectively. The Chi-square statistic (χ^2) for the 2×2 table is computed by the following formula and compared to 1 degree of freedom at the 0.05 type I error rate.

$$\chi^2 = \frac{N_{..}(n_{11}n_{22} - n_{12}n_{21})^2}{N_{1.}N_{2.}N_{.1}N_{.2}}$$

In general, Chi-square analysis requires an expected cell count ≥ 5 . To be conservative, when any of the expected count values in the 2×2 table (n_{11} , n_{12} , n_{21} , n_{22}) are less than or equal to 10, we performed the Fisher exact test in addition to the Chi-square test. In the Fisher exact test, we fixed the marginal sum (i.e. $N_{1.}$, $N_{2.}$, $N_{.1}$, $N_{.2}$) and created all possible tables with the same marginal sum. The probability of observing each possible table configuration is computed as below.

$$P = \frac{N_{1.}! N_{2.}! N_{.1}! N_{.2}!}{N_{..}! n_{11}! n_{12}! n_{21}! n_{22}!}$$

The two-sided p-value of the Fisher exact test is the sum of all probabilities that are less than or equal to the probability derived from the original observed table. We used these methods to compare SAGE tag abundance for each pair of AD and control libraries.

Since p-values were calculated for several thousands of genes simultaneously, we applied Benjamini and Hochberg's (Benjamini and Hochberg, 1995) false discovery rate (FDR) procedure to correct for multiple testing. We simultaneously test n null hypotheses H_1 , H_2 , ..., H_n on the basis of independent chi-square or Fisher exact tests. The p-values were

ordered as $P_{(1)} \leq P_{(2)} \leq \dots \leq P_{(n)}$. If k is the largest integer such that $P_{(k)} \leq \frac{k}{n} \alpha$, then we reject all H_i for $i \leq k$, where $p_o =$ proportion of true H_i . For this analysis, we assume $\alpha = 0.05$ and $p_o = 1$ (i.e. all H_i are true). We have used two different values for the number of independent tests.

Functional categorization

We assigned differentially expressed gene transcripts to functional categories by two methods. One method assigned transcripts to broad functional categories defined on the basis of gene function derived from literature searches. The other method used the software tool, the Expression Analysis Systematic Explorer (EASE; <http://david.abcc.ncifcrf.gov>), to assign identified gene transcripts to "GO: molecular function" categories of the GeneOntology Consortium (www.geneontology.org). We used the GO system to quantify over- and under-represented (up- and down-regulation) transcripts relative to total genes within a functional category and compare between different SAGE libraries. Since the GO system does not provide a functional designation for all genes, our functional interpretations relied primarily on the extensive literature search in PubMed and sequence search in NCBI and other HGP databases.

Quantitative real-time PCR

We confirmed selected SAGE data in individual *APOE* allele-specific ADs and controls by quantitative real-time PCR (qRT-PCR). Hippocampal total RNA (10 ug) was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Assays-on Demand™ Gene Expression primer sets for qRT-PCR were purchased from Applied Biosystems (Table 6). The primer sequences can be obtained from the authors upon request. Four housekeeping genes (GAPD, GUSB, HPRT1, APLPO) were used as endogenous controls (or references). Real-time PCR was performed using TaqMan Universal PCR Master Mix according to TaqMan Gene Expression Assay protocol (ABI). Each reaction was performed in quadruplicates, and non-RT (without reverse transcriptase) and non-RNA template controls were included. The relative amounts of specifically amplified cDNAs in *APOE* allele-specific ADs compared with *APOE3/3* controls were calculated using comparative C_T method ($\Delta\Delta C_T$ method) using Arithmetic

Formula: $2^{-\Delta\Delta C_T}$ (User Bulletin #2, ABI). The average C_T value for each genotype of AD cases was calculated from individual samples in each *APOE* genotype group (Table 1) on four independent experimental replicates. The mean ΔC_T value was calculated by formula: $\Delta C_T = C_T \text{ target} - C_T \text{ reference}$. The $\Delta\Delta C_T$ of target gene was calculated by formula: $\Delta\Delta C_T = \Delta C_T \text{ target} - \Delta C_T \text{ calibrator}$. We use average GAPDH C_T value as C_T reference and average ΔC_T value of *APOE3/3* control as ΔC_T calibrator.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Scatter plots showing comparative expression levels of tags between different pairs of libraries. (A) SAGE tag frequency distribution of *APOE3/3* AD vs. *APOE3/3* control, (B) *APOE3/4* AD vs. *APOE3/3* control, and (C) *APOE4/4* AD vs. *APOE3/3* control. (D) Comparisons of SAGE tag count between *APOE3/4* AD vs. *APOE3/3* AD, (E) *APOE4/4* AD vs. *APOE3/3* AD, and (F) *APOE4/4* AD vs. *APOE3/4* AD.

Figure 2.

Quantitative differences of up- and down-regulated ($P < 0.05$) SAGE tags mapped to UniGene clusters compared between two libraries. (A) Total tags compared between two libraries using eSAGE software; (B) Significant tags showing changes greater than two-fold ($P < 0.05$) between two libraries; (C) Total mapped UniGene clusters showing up- and down-regulation in comparisons of two libraries. (D) Unique UniGenes showing up- and down-regulation in the comparisons of two libraries.

Figure 3. Molecular functional categories of genes up-regulated (**A**) and down-regulated (**B**) in *APOE3/3* AD, *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* control. The software tool, expression analysis systematic explorer (EASE; <http://david.niaid.nih.gov/david/ease.htm>), was used to assign identified genes to molecular function categories of the Gene Ontology Consortium (www.geneontology.org).

Figure 4.

Comparison of molecular functional categories of gene transcripts up-regulated (**A**) and down-regulated (**B**) in *APOE4/4* and *APOE3/4* cases compared with *APOE3/3* AD hippocampus, or *APOE4/4* AD compared with *APOE3/4* AD cases. EASE was used to assign identified genes to molecular function categories of the Gene Ontology Consortium.

Figure 5. Regulation of gene expression by *APOE* genotype: comparison of effects of the *APOE3/3*, *APOE3/4* and *APOE4/4* alleles on AD versus *APOE3/3* control samples. **(A)** Venn diagram showing the number of genes that shared up-regulation between *APOE3/3* AD, *APOE3/4* AD and *APOE4/4* AD compared with control. **(B)** Venn diagram showing the number of genes that shared down-regulation between *APOE3/3* AD, *APOE3/4* AD and *APOE4/4* AD compared with control. Full list of overlapped genes are included in Supplementary Table 1-3 online.

Figure 6.

Comparison of gene expression changes between *APOE4/4* AD, *APOE3/4* AD and *APOE3/3* AD. **(A)** Venn diagram showing the number of up-regulated genes in *APOE4/4* AD, *APOE3/4* AD and *APOE3/3* AD with shared up-regulation of *APOE3/4* AD vs. *APOE3/3* AD, *APOE4/4* AD vs. *APOE3/3* AD, and *APOE4/4* AD vs. *APOE3/4* AD. **(B)** Venn diagram showing the number of down-regulated genes in *APOE4/4* AD, *APOE3/4* AD and *APOE3/3* AD with shared down-regulation of *APOE3/4* AD vs. *APOE3/3* AD, *APOE4/4* AD vs. *APOE3/3* AD, and *APOE4/4* AD vs. *APOE3/4* AD. Full list of overlapped genes are included in Supplementary Table 4-6 online.

A

Up-regulated

E34 AD/E33 AD E34 AD/E33 C

E44 AD/E33 AD E44 AD/E33 C

B

Down-regulated

E34 AD/E33 AD E34 AD/E33 C

E44 AD/E33 AD E44 AD/E33 C

Figure 7.

Overlapped genes in the comparisons of *APOE4/4* AD and *APOE3/4* AD compared with *APOE3/3* AD and *APOE3/3* control. (A) Venn diagram showing the number of overlapped up-regulated genes in *APOE4/4* AD and *APOE3/4* AD vs. *APOE3/3* AD and *APOE3/3* control. (B) Venn diagram showing the number of overlapped down-regulated genes in *APOE4/4* AD and *APOE3/4* AD vs. *APOE3/3* AD and *APOE3/3* control. Full list of overlapped genes are included in Supplementary Table 7-8 online.

Table 1
Summary of clinical and pathological data of hippocampal samples used for generation of SAGE libraries and RT-PCR analyses

Sample ID	Age (Years)	Sex (M/F)	PMD (hr:min)	Pathology B&B stage	SAGE Library	RT-PCR analyses
<i>APOE3/3</i> Control						
542	81.0	M	3:15	B&B I		Yes
673	80.7	F	1:10	B&B I	Yes	Yes
707	80.8	M	4:15	B&B I		No*
787	93.1	F	10:38	B&B I		Yes
<i>APOE3/3</i> AD						
470	95.0	F	1:01	B&B IV	Yes	Yes
589	90.0	F	2:20	B&B IV		Yes
721	65.0	F	3:36	B&B V		Yes
792	65.8	F	17:28	B&B V		No
214		M	17:00	B&B IV		Yes
<i>APOE3/4</i> AD						
648	82.0	F	2:15	B&B V		Yes
706	90.5	F	9:30	B&B IV		No
723	64.7	M	0:43	B&B V		Yes
724	86.9	F	1:30	B&B IV	Yes	Yes
211		M	8:15	B&B IV		Yes
213	84.0	M	16:00	B&B IV		Yes
<i>APOE4/4</i> AD						
545	76.5	F	3:25	B&B V		Yes
616	62.4	F	2:10	B&B V		Yes
957	83.2	M	2:57	B&B IV	Yes	Yes
977	80.0	M	6:40	B&B V		Yes
2137	76.0	M	22:20	B&B IV		Yes

PMD: postmortem delay.

B&B stage: Braak & Braak stage.

* Samples not included in RT-PCR analysis because of RNA degradation.

Table 2

Characterization of SAGE libraries

SAGE library	Total tags sequenced	Unique tags identified	UniGenes mapped	Unique UniGenes	Orphan tags
<i>APOE3/3</i> control	70,456	23,126	23,373	13,421	5,398 (18.8%)
<i>APOE3/3</i> AD	78,126	25,140	25,088	14,503	6,031 (19.4%)
<i>APOE3/4</i> AD	72,095	24,663	23,984	13,791	6,468 (21.2%)
<i>APOE4/4</i> AD	67,984	22,167	21,884	12,577	5,063 (20.4%)
Total	288,661	95,096	94,329	54,292	22,960 (19.6%)

Unique tags: tags with different tag sequence identified in total extracted tags.
Unique UniGene: UniGene cluster that maps to one location in the genome.
Orphan tags: tags that do not map to any known UniGene cluster.

Table 3
Summary of differentially ($p < 0.05$) expressed SAGE tags mapped to UniGenes compared between different SAGE libraries

SAGE library	Total tags compared	Significant tags		Total UniGenes			Unique UniGenes		
		N	%	Total	Up	Down	Total	Up	Down
E33AD vs. E33C	37,900	156	0.41	276	114	162	100	48	52
E34AD vs. E33C	39,059	906	2.32	1594	714	880	523	97	426
E44AD vs. E33C	36,632	625	1.71	1270	847	423	320	108	212
E34AD vs. E33AD	40,584	918	2.26	1673	848	825	499	131	368
E44AD vs. E33AD	38,358	771	2.01	1469	934	535	428	172	256
E44AD vs. E34AD	38,283	476	1.24	761	533	208	287	188	99

Total tags: eSAGE software compared databases.

Total UniGenes: significant tags mapped to UniGene clusters.

Unique UniGenes: tags that map to one UniGene cluster.

Table 4
Gene Ontology: Quantitative differences of selected categories of up- and down-regulated genes in *APOE* allele-specific AD compared with *APOE3/3* control

Molecular function	Up-regulated genes			Down-regulated genes		
	<i>APOE33AD</i>	<i>APOE34AD</i>	<i>APOE44AD</i>	<i>APOE33AD</i>	<i>APOE34AD</i>	<i>APOE44AD</i>
Protein binding & transporter	6	13	9	7	46	31
Receptor binding & signaling	3	7	4	3	24	13
Metal ion binding	5	6	6	1	30	20
Nucleotide binding	4	7	8	4	31	21
Nucleic acid binding	8	8	5	5	40	22
Hydrolase activity	5	2	10	4	40	21
Ion transporter		2	1	1	21	13
Transferase activity	1	4	5	4	20	10
Electron & channel transporter	2	1	3		16	6
Carrier activity		4	2	2	17	12
Kinase activity	1	3	5	3	14	8
Enzyme activator & inhibitor	1	4	1	1	10	5
Oxidoreductase	2	1	4		9	5
Transcription factor	2	2	2	2	9	12

Table 5
Gene Ontology: Quantitative differences of selected categories of up- and down-regulated genes between *APOE* allele-specific AD

Molecular function	Up-regulated genes				Down-regulated genes			
	APOE34AD/ APOE33AD	APOE44AD/ APOE33AD	APOE44AD/ APOE34AD	APOE44AD/ APOE34AD	APOE34AD/ APOE33AD	APOE44AD/ APOE33AD	APOE44AD/ APOE34AD	APOE44AD/ APOE34AD
Protein binding & transporter	17	18	26	39	46	39	9	9
Receptor binding & signaling	7	7	13	16	27	16	4	4
Metal ion binding	6	5	17	25	27	25	9	9
Nucleotide binding	10	9	21	19	36	19	5	5
Nucleic acid binding	3	12	10	28	36	28	7	7
Hydrolase activity	3	12	20	17	26	17	3	3
Ion transporter activity	2	1	6	13	17	13	1	1
Transferase activity	7	11	12	11	24	11	2	2
Electron & channel transporter	2	2	4	10	16	10	1	1
Carrier activity	6	5	6	13	12	13	5	5
Kinase activity	7	7	11	13	17	13	2	2
Enzyme activator & inhibitor	5	2	5	3	5	3	2	2
Oxidoreductase	3	3	2	10	8	10	1	1
Transcription factor	1	1		16	16	16	3	3

Table 6
Examples of genes differentially expressed in *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* control verified by qRT-PCR

SAGE			qRT-PCR							
SAGE tag	Gene	Gene ID	E34AD/E33C				E44AD/E33C			
			E34 AD	E33 C	Fold		E44 AD	E33 C	Fold	
Upregulated genes										
CATATCATTA	IGFBP7	3490	33	5	6.5				2.31	1.23
ACGCAGGGAG	MALAT1	29005	61	6	10	66	6	11.5	1.29	1.89
ATGAAACTTC	DLC1	10395	6	0	5.9	5	0	5.2	1.46	1.81
TAAACCAAGAG	TTR	7276	1466	0	1466				38.29	3.92
CCGTGCTCAT	DCXR	51181	9	1	8.8	7	1	7.3	1.04	1.54
GACTCTTCAG	SERPINA	12	21	1	20.6				1.6	-1.04
GTGGCAGGTG	CD24	934	166	96	1.7	133	96	1.4	2.12	-1.63
Downregulated genes										
TCAATCAAGA	YWHAH	7533	3	56	-19	25	56	-2.2	-2.15	-1.52
TGCTTGATAG	RGS4	5999	1	29	-29.6	9	29	-3.1	-6.76	-3.74
GTGCGAATCC	CAMK2A	815	1	17	-17.4	1	17	-16.3	-1.98	-1.45
ATCCGTGCCC	CALM3	808	4	48	-12.3	5	48	-9.2	-3.16	-2.17
AAGCACATTA	SLC1A2	6506	1	13	-13.3	1	13	-12.5	-1.66	-1.61
ACAACACTAC	RAB3A	5864	0	21	-21	6	21	-3.4	-2.99	-2.08
GGCCCTCTGA	PIN1	5300	8	45	-5.7	26	45	-1.7	-1.02	1.06
CAGTAGTACA	A2BP1	54715	0	11	-11.2	2	11	-5.3	-1.94	-1.55
TGACAGTGAC	OXR1	55074	1	7	-7.1	0	7	-6.7	-2.18	-1.32

* RT-PCR fold differences were calculated from the average C_T values of *APOE3/4* and *APOE4/4* AD samples compared with the average C_T values of *APOE3/3* controls.

Table 7

Functional cluaters of genes 5-fold up-regulated in *APOE3/3*, *E3/4* and *E4/4* ADs compared with *APOE3/3* control

<i>APOE3/3</i> AD vs. <i>APOE3/3</i> C					<i>APOE3/4</i> AD vs. <i>APOE3/3</i> C					<i>APOE4/4</i> AD vs. <i>APOE3/3</i> C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene	Tag	E44 AD	E33 Ctrl	Fold	Gene
Signal transduction and G-protein signaling														
CAGGCCCAAA	6	0	5.4	MAP3K12	ATGTAGTGC	8	1	7.8	PPAP2B	GCCCCCACT	5	0	5.2	MAPKAPK2
GCCCCCTGC	6	0	5.4	PLEKHJ1	TTAGATCGTT	6	0	5.9	TM4SF6	TATTTGTGA	7	1	7.3	MARCKS
					ACGCGCCAGG	8	0	7.8	EF5	ACTAAAAACAC	5	0	5.2	FLJ23091
					GACAGTTAGG	10	1	9.8	DOCK5	AACCCCGGA	7	1	7.3	PRKCQ
									ATGACTTGTG		7	1	7.3	CAP2
									GGGAGACCTC		5	0	5.2	DDAH2
Ca++ signaling and homeostasis														
GGCTTGGTTT	10	1	9	CAMTA2	AGAAGAGGCT	6	0	5.9	CAPS2	GGCTTGGTTT	9	1	9.4	CAMTA2
TATTTTGTG	8	0	7.2	EFCBP1	CAAGGTAAG	11	2	5.4	EFEMP1	GAGACGGCGC	5	0	5.2	SMOC1
AGATTGAGGC	11	2	5	INPP5F	CTGACCAGAG	12	1	11.8	CAPS					
Cell adhesion and myelinogenesis														
ATTGCCAACA	9	1	8.1	MOBP	GCCACTCTGC	9	0	8.8	CEECAM1	AGCTTGTTC	5	0	5.2	NRCAM
TTACATTGTA	12	2	5.4	OPCML	GGGCAGTGGC	9	0	8.8	CIQTNF5	GGCACGGACG	6	0	6.2	CTNND2
					GTTTTCCTGA	10	1	9.8	CLDN11					
					TGCCGTGCGG	10	1	9.8	FA2H					
					GACAGCCATC	6	0	5.9	CLMN					
Cell growth and proliferation														
CTGTGACACA	11	2	5	CCT2	AGAAATCGCCT	6	0	5.9	DHX33	TGATTGGTGG	7	0	7.3	PDGFRA
TGCGGAGGCC	8	1	7.2	SSSCA1	AGCCTTGTG	7	0	6.9	SERPINH1	AACGCCAACA	7	0	7.3	SART1
					AGGCTGGATG	23	3	7.5	UNC84B					
Tumor suppressor and cell arrest														

APOE3/3 AD vs. APOE3/3C					APOE3/4 AD vs. APOE3/3 C					APOE4/4 AD vs. APOE3/3 C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene	Tag	E44 AD	E33 Ctrl	Fold	Gene
Tumorigenesis														
Transcription factor and regulator														
Protein modification														
Oxidoreductase														

Table 8

Clusters of genes 5-fold up-regulated in *APOE3/4* AD and *APOE4/4* AD compared with *APOE3/3* AD

APOE34 AD vs. APOE33 AD						APOE44 AD vs. APOE33 AD					
Tag	E34 AD	E33 AD	Fold	Gene	Gene ID	Tag	E44 AD	E33 AD	Fold	Gene	Gene ID
Signal transduction											
TTAGATCGTT	6	0	6.5	TM4SF6	7105	ACGTCAGGAG	5	0	5.8	MAP3K7IP1	10454
CTGACCAGAG	12	0	13	CAPS	828	CAAAATCCAAA	6	0	6.9	MAP4K3	8491
ACGGCGCCA	5	0	5.4	SFRP1	6422	GAATCATTTT	11	2	6.4	ARL6IP	23204
GCCTGGTGTG	5	0	5.4	CDC42BPA	8476	AACCCCGGGA	7	0	8.1	PRKCQ	5588
ACGTCAGGAG	5	0	5.4	MAP3K7IP1	10454	GGGACACGGCA	5	0	5.8	KNDC1	85442
AACCCCGGA	7	0	7.6	PRKCQ	5588	AAACATTAGC	7	1	8.1	PTP4A2	8073
ACGCGCCAGG	8	0	8.7	EFS	10278	TAITTTGTGA	7	0	8.1	MARCKS	4082
CAAGGGTAAG	11	1	11.9	EFEMP1	2202	AGCACTGCAG	15	1	17.3	NMT1	113026
GACAGTTAGG	10	2	5.4	DOCK5	80005	GCACTGTGCT	13	2	7.5	PDE4DIP	9659
GTGGGCGTTG	10	2	5.4	PSMD2	5708	CCATCACACT	6	0	6.9	TRPC4AP	26133
ACCGGGGTGA	5	0	5.4	GNA12	441190	GTGGTGCAGG	5	0	5.8	RGS6	9628
						GAGCAGCGGG	5	0	5.8	LGR4	55366
Cell adhesion and myelinogenesis											
GCCACTCTGC	9	1	9.8	CEECAM1	51148	GGCACGGAGC	6	0	6.9	CTNND2	1501
GTTTTCCTGA	10	1	10.9	CLDN11	5010	TGAAACCCCTG	13	1	15	PARD3	56288
CTCGCGCTGG	5	0	5.4	CLDN3	1365	ACGCAGAGAC	5	0	5.8	KIF1A	547
GGGCAGTGGC	9	0	9.8	C1QTNF5	114902	TTTCATCCAC	7	1	8.1	MCF2L	23263
TGCCTGTCCG	10	1	10.9	FA2H	79152						
CCAGGCTGGC	7	1	7.6	HAPLN2	60484						
TGAAACCCCG	13	2	7.1	TMEM23	259230						
GACAGCCATC	6	0	6.5	CLMN	79789						
TGAAACCCCTG	11	1	11.9	PARD3	56288						
GCCTGTCCCT	5	0	5.4	BGN	633						
Tumor suppressor and cell arrest											
GATAACTACA	5	0	5.4	IGFBP7	3490	GCAATGAGCT	13	2	7.5	MEG3	55384
TGACCAAGAG	7	0	7.6	MCC	4163	GGCCAGCAAG	5	0	5.8	MUTYH	4595

APOE34 AD vs. APOE33 AD						APOE44 AD vs. APOE33 AD					
Tag	E34 AD	E33 AD	Fold	Gene	Gene ID	Tag	E44 AD	E33 AD	Fold	Gene	Gene ID
Tumorigenesis											
CTGCTCATAA	6	0	6.5	HIPK2	28996	GGCCAGGTGG	10	1	11.6	PCOLN3	5119
GAAACAGACG	7	1	7.6	ELAC2	60528	ACGCAGGGAG	66	9	8.5	MALAT1	378938
GGAATGCCTC	14	1	15.2	ECRG4	84417						
ACGCAGGGAG	61	9	7.4	MALAT1	29005						
CGACGAGGAG	6	0	6.5	EMP3	2014						
CTTCCTGGCC	21	0	22.8	XIST	7503						
Protein synthesis											
CCTTTGGCTA	5	0	5.4	RPS27L	51065	TCTGGGGACG	7	0	8.1	EIF2S2	8894
ATACTGAAGC	5	0	5.4	MRPS6	64968	ACAGAGCGAG	19	4	5.5	RPL13	6137
TAGGCCCAAG	5	0	5.4	ILVBL	10994						
TCTCCAACAA	6	0	6.5	HPS3	84343						
Protein modification											
ACTGCCCAA	5	0	5.4	SSP411	64847	ATTGCAGCAC	6	0	6.9	LCMT1	51451
TTCACATTAG	5	0	5.4	CCT4	10575	TCCCCTACAT	5	0	5.8	ADAMTS16	170690
						GTGAGACACT	5	0	5.8	AK3L1	50808
						CTGGGAGAGG	7	1	8.1	RNPEPL1	57140
						CTTTCAGATG	16	3	6.2	PFKP	5214
						ATCTGCCAC	7	0	8.1	GBA2	57704
						GAAGAGTCTC	6	0	6.9	USP36	57602

Selected 6 major 5-fold upregulated gene categories were listed. Full lists of the genes are included in Supplementary Table 12-13.

Table 9

Multiple genes of synaptic plasticity and neuronal outgrowth are downregulated in *APOE3/4* and *APOE4/4* ADs vs. control

APOE3/3 AD vs. APOE3/3C				APOE3/4 AD vs. APOE3/3 C				APOE4/4 AD vs. APOE3/3 C					
Tag	E33 AD	E33 Ctrl	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene	Tag	E44 AD	E33 Ctrl	Fold	Gene
Plasticity and synaptic formation													
				TCTAGATGCA	0	5	-5.1	DLGAP1	GCCCCAGCTG	6	46	-7.4	GRIN1
				TTGTGAGAAAT	0	6	-6.1	DLGAP4	GTGGCGGGAT	1	8	-7.7	EFNB3
				CCCCGCAGCT	1	29	-30	PPP3CA	TCCTGGTGC	1	8	-7.7	SYNGR1
				TCCTGTGTCG	0	8	-8.2	SYNGR1	TGGTTTCACAT	0	7	-6.7	NPTX1
				TTCCGACTGC	0	14	-14	SYNGR3	CCCCGCAGCT	4	29	-7	PPP3CA
				TTCTCAATAC	1	11	-11	SYNPO					
				TACGCCCTCT	9	46	-5.2	SNAP91					
				AGAGTAATCA	1	14	-14	GAP43					
				GTGGCGGGAT	0	8	-8.2	EFNB3					
				TATTATCTTG	0	5	-5.1	PTPN5					
				TGGTTCACAT	1	7	-7.1	NPTX1					
				TGGACAAAGCT	0	6	-6.1	BASP1					
				CTTATGACAA	0	11	-11	CHGB					
Synaptic vesicle docking and fusing													
				ATTAAAGTCA	0	30	-31	RIMS3	CTGTGTCCAA	2	15	-7.2	EPS15
				GATCACAAT	0	5	-5.1	RIMS2	TCGTAAGGCA	1	9	-8.6	PPFIA4
				TGCACAGTTA	2	15	-7.7	RIMBP2	TAGTTTACAC	0	6	-5.8	TRIM9
				TACCCCTCTG	2	26	-13	SYT1	ATCTTCTCGG	1	11	-11	YWHAZ
				AGACATTGTA	2	20	-10	SYT13					
				CTGGACGTAG	2	20	-10	SV2A					
				GGGGTGCTGT	10	105	-11	DNM1					
				ACAACACTAC	0	21	-21	RAB3A					
				CACAACCACC	0	21	-21	RPH3A					
				TGGAGGTGGG	0	5	-5.1	VAPB					

APOE3/3 AD vs. APOE3/3C					APOE3/4 AD vs. APOE3/3 C					APOE4/4 AD vs. APOE3/3 C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene	Tag	E44 AD	E33 Ctrl	Fold	Gene
					TCGGGGCCCC	4	28	-7.1	CPLX1					
					CTGTGTCCAA	3	15	-5.1	EPS15					
					TAGTTTACAC	0	6	-6.1	TRIM9					
					TCGTAAAGGCA	1	9	-9.2	PPFIA4					
					TCAATCAAGA	3	56	-19	YWHAH					
					ATCTTCTGG	1	11	-11	YWHAZ					
					AATGTGAGTC	8	47	-6	YWHAG					
Axonal guidance, neuronal outgrowth and neurogenesis														
TTTACAGGGT	1	7	-7.8	RTN4R	TGCTGAGGGT	1	12	-12	DOK6	TGATGTTAGT	0	7	-6.7	CDH10
					GCAGGCCTGC	1	18	-18	NGEF	GCAGGCCTGC	2	18	-8.6	NGEF
					ACATTGAGTC	0	5	-5.1	SLIT1	TAAAAGAGCC	0	7	-6.7	GAS7
					TGATGTTAGT	0	7	-7.1	CDH10	TCACCTTAGG	4	22	-5.3	ITM2B
					TGACTGCTGC	1	15	-15	GSK3B	TTTTTACTGA	2	13	-6.2	ITM2C
					TAACTTAAG	1	31	-32	OLFM1	GAATGCTGAC	0	6	-5.8	TPP1
					CATTATAACT	0	5	-5.1	DULLARD	GCAGGAACAG	2	12	-5.8	KIAA1068
					CATTCTCTACT	0	5	-5.1	AF1Q	CATTGAGACA	0	10	-9.6	ELAVL3
					CCAAACAAGAA	3	37	-13	TM4SF2					
					GAGTGAGACC	3	30	-10	THY1					
					CCCTCTGTCA	1	8	-8.2	PPP1R9B					
					TGCGGCTGGT	2	13	-6.6	DCTN1					
					CATTGAGACA	0	10	-10	ELAVL3					
					TTTACAGGGT	0	7	-7.1	RTN4R					
					CTGAGGGGTG	0	5	-5.1	MAP1LC3B					

Table 10

Multiple genes regulating signal transduction and calcium homeostasis are downregulated in ADs compared with control

APOE3/3 AD vs. APOE3/3C					APOE3/4 AD vs. APOE3/3 C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene
Signal transduction									
ACGTCAGGAG	0	8	-8.9	MAP3K7IP1	TCCAGGTCTC	1	13	-13	MAPK9
AGCAAAATATG	1	7	-7.8	PRKACB	GCCTCAATAA	1	9	-9.2	MAPK8IP2
ACGAGTCATT	1	7	-7.8	SEZ6L	CCTACATTTT	1	17	-17	CHN1
GCCTGGTGTG	0	9	-10	CDC42BPA	CGCTGCTCAA	1	16	-16	PTPRN
					CACTTCAAGG	2	23	-12	LY6E
					GAGACTCTTA	1	9	-9.2	HABP4
					AAGCATTAAA	1	10	-10	PRKAR1B
					AGCAAAATATG	0	7	-7.1	PRKACB
					AAATAGGTTT	0	7	-7.1	PARK7
					TAGCTGCCTT	0	6	-6.1	DVL2
					CTGGCGCTGA	2	11	-5.6	SHC2
					ACCCCAACCCA	2	11	-5.6	AGPAT1
					GTCTCTACGA	0	5	-5.1	TAGLN3
					GGAGGGATCA	2	10	-5.1	ILK
					ATAATGGAGT	0	5	-5.1	BTRC
					ACGAGTCATT	0	7	-7.1	SEZ6L

G-protein signaling									
AGGAGGCGGA	0	5	-5.5	RANBP1	GACAATGTAT	1	7	-7.1	GNG2
					TGAGGTTATC	0	7	-7.1	GNG3
					CACCTAGGGG	0	5	-5.1	GNB5
					ACCAATCCTA	1	11	-11	GNAO1
					TGCTTGATAG	1	29	-30	RGS4
					AGGAGGCGGA	0	5	-5.1	RANBP1
					TCTGGCAGTA	1	7	-7.1	RANBP5

Neurotransmitter receptors and genes involved in cell protection, mitochondrial oxidative phosphorylation and cytoskeleton are downregulated in ADs vs. control

Table 11

APOE3/3 AD vs. APOE3/3C					APOE3/4 AD vs. APOE3/3 C					APOE4/4 AD vs. APOE3/3 C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 AD	Fold	Gene	Tag	E44 AD	E33 AD	Fold	Gene
Neurotransmitter receptors and signaling														
AAAGGCGCTG	0	6	-6.7	SLC25A18	ACTGGACTTA	1	11	-11.2	GABRA5	TACTACCCTC	3	23	-7.4	GABBR1
					CAACAGGCCT	1	8	-8.2	SLC6A7	ACTGGACTTA	1	11	-11	GABRA5
					GTCCCACTTC	0	8	-8.2	SLC6A1	TGTACTTCCT	0	6	-5.8	OAT
					TGTACTTCCT	0	6	-6.1	OAT	GTCCCACTTC	0	8	-7.7	SLC6A1
					TTCTTACAAA	0	5	-5.1	GRIA2	CAACAGGCCT	1	8	-7.7	SLC6A7
					CCACACCCCT	0	5	-5.1	GRIK2	AAGCACATTA	1	13	-13	SLC1A2
					GAGAAATTGA	1	7	-7.1	GAD1					
					AAGCACATTA	1	13	-13.3	SLC1A2					
Cell protection and chaperone														
TAACTCTCCT	0	5	-5.5	SCARA3	CAGGTAAGGT	0	10	-10.2	DNAI4	TGATTTCACT	31	220	-6.8	HSPCB
CAGAGATGAA	0	6	-6.7	HSPA1A	TCCTAGCCTG	1	9	-9.2	DNAJC8	TGACAGTGAC	0	7	-6.7	OXRI
GTGGGGGGAG	0	9	-10	HKE2	TTCTCTTGGA	0	10	-10.2	STCH	CTCTTCGAGA	1	8	-7.7	GPX1
					CATTGAAGGG	2	16	-8.2	MLF2					
					TGACAGTGAC	1	7	-7.1	OXRI					
					ACTGATTGAT	0	5	-5.1	AHSA2					
Mitochondria proteins and oxidative phosphorylation														
					CATTTCATAA	2	13	-6.6	ATP5J	GATCCCAACA	1	17	-16	ATP5B
					TGAGAGGAGA	0	6	-6.1	NDUF5	TTTGCCTGTT	2	11	-5.3	ATP6VIH
					TTTGCTTGTT	1	11	-11.2	ATP6VIH	TAAATGGTAAC	1	10	-9.6	COX5A
					TGTGATCAGA	2	20	-10.2	ATP5L	GACATAATGTA	1	10	-9.6	COX7B
					TAATGGTAAC	0	10	-10.2	COX5A	AAAGGCGCTG	0	6	-5.8	SLC25A18
					AATATGCTTT	1	8	-8.2	ATP6VIE1	CAACTGTATT	1	8	-7.7	ACO2
					CGGATGATTG	1	7	-7.1	SLC25A11					

APOE3/3 AD vs. APOE3/3C					APOE3/4 AD vs. APOE3/3 C					APOE4/4 AD vs. APOE3/3 C				
Tag	E33 AD	E33 Ctrl	Fold	Gene	Tag	E34 AD	E33 Ctrl	Fold	Gene	Tag	E44 AD	E33 Ctrl	Fold	Gene
					GTTCTGGTTT	3	20	-6.8	ATPIF1					
					TGGCTGTGAG	5	27	-5.5	ATP6V0A1					
					CTGTTAGTGT	9	95	-10.8	MDHI					
					ATGGAGACTT	1	7	-7.1	CS					
					ATTCTGTTCAT	1	7	-7.1	SUCLA2					
					TAAATTCACC	0	5	-5.1	TOMM70A					
Cytoskeleton and sturcture and stability														
					AATGTTTGGC	1	10	-10.2	3-Sep	GCTTTATTTG	1	19	-18	ACTB
					CCATCCAGGC	1	10	-10.2	SNX10	ACCTTACAGT	0	15	-14	PFN2
					TGTAGAAAAA	1	17	-17.4	TUBB2	CGGCTTTTCT	1	15	-14	SPTBN1
					ACCTTACAGT	1	15	-15.3	PFN2	GTAGAATAGT	0	6	-5.8	MAPRE2
					TGCCGCACGT	2	24	-12.3	KNS2					
					TACGCCAAGC	1	9	-9.2	TUBA8					
					TTGCTATGAA	1	7	-7.1	KIF21A					
					GTAAGTCTCA	3	20	-6.8	NEFL					
					CTTCAATAGT	0	8	-8.2	INA					
					AGCACATTTG	0	6	-6.1	COTL1					
					GGAGAAAGATG	0	6	-6.1	PFDN2					
					GTACCCGTAC	1	13	-13.3	PDXP					
					TAACTTACAT	1	8	-8.2	WASF1					
					AACAGAAAGTG	0	5	-5.1	FARP1					
					GTGATACGTT	0	5	-5.1	AFAP					

Full list of genes are included in Supplementary Table 9-11.