Cumulative effects of the ApoE genotype and gender on the synaptic proteome and oxidative stress in the mouse brain

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Abstract

Elderly females, particularly those carrying the apolipoprotein E (ApoE)-ε4 allele, have a higher risk of developing Alzheimer’s disease (AD). However, the underlying mechanism for this increased susceptibility remains unclear. In this study, we investigated the effects of the ApoE genotype and gender on the proteome of synaptosomes. We isolated synaptosomes and used label-free quantitative proteomics, to report, for the first time, that the synaptosomal proteomic profiles in the cortex of female human-ApoE4 mice exhibited significantly reduced expression of proteins related to energy metabolism, which was accompanied by increased levels of oxidative stress. In addition, we also first demonstrated that the proteomic response in synaptic termini was more susceptible than that in the soma to the adverse effects induced by genders and genotypes. This suggests that synaptic mitochondria might be ‘older’ than mitochondria in the soma of neurons; therefore, they might contain increased cumulative damage from oxidative stress. Furthermore, female human-ApoE4 mice had much lower oestrogen levels in the cortex and treatment with oestrogen protected ApoE3 stable transfected C6 neurons from oxidative stress. Overall, this study reveals complex ApoE- and gender-dependent effects on synaptic function and also provides a basis for future studies of candidates based on specific pathways involved in the pathogenesis of AD. The lack of oestrogen-mediated protection regulated by the ApoE genotype led to synaptic mitochondrial dysfunction and increased oxidative stress, which might make older females more susceptible to AD.

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Introduction

The apolipoprotein E (ApoE) ε4 allele is considered to be a risk factor for developing sporadic Alzheimer’s disease (AD) (Kim et al., 2009; Chen et al., 2011a). Genome-wide association studies (GWAS) have consistently identified ApoE as an unequivocal susceptibility gene in AD (Avramopoulos, 2009; Harold et al., 2009). ApoE was also strongly associated with the rate of cognitive decline in a GWAS using a quantitative measure of global cognitive decline (De Jager et al., 2012). We demonstrated previously that mild cognitive impairment (MCI) in patients was associated with a higher frequency of the ApoE4 allele. Moreover, there was a significant decline in olfactory identification in MCI subjects bearing the ApoE ε4 allele (Wang et al., 2002). The ApoE 4 carriers with MCI also had significantly reduced cerebral blood flow velocity (Sun et al., 2007).

The presence of one or two ApoE4 alleles has been associated with an increased rate of progression from MCI to AD (Petersen et al., 1995). Numerous studies have revealed that ApoE4 significantly impaired synaptic and mitochondrial function, which might be an early event in the onset of AD. For example, ApoE4 decreased spine density, affected dendritic complexity (Ji et al., 2003; Dumanis et al., 2009), and caused mitochondrial dysfunction (Valla et al., 2010; Chen et al., 2011a) and oxidative damage (Jofre-Monseny et al., 2008) in humans and transgenic mouse models of AD. Greicius and colleagues used functional magnetic resonance imaging, and found weaker brain connectivity in the precuneus and posterior cingulate cortex of female ApoE4 carriers compared with either female ApoE3 homozygotes or male carriers (Damoiseaux et al., 2012). Therefore, there are gender differences in the magnitude of the effect of this allele...
in the brain. Interestingly, it is generally recognized that elderly females, particularly those carrying ApoE4 allele, have a higher risk of developing AD than males (Payami et al., 1996) and are also more sensitive to the deleterious effects of ApoE4 on cognition (Mortensen and Hogh, 2001).

It is possible that oestrogen deficiency in the brains of post-menopausal females might increase the risk of developing AD (Yue et al., 2005). There is a close relationship between oestrogen and ApoE in the central nervous system (Nathan et al., 2004), where the role of oestrogen in neural protection and synaptic plasticity might be modulated by the ApoE genotype (Stone et al., 1998). In addition, oestrogen replacement therapy (ERT) might reduce the risk of AD in the absence of ApoE4 (Yaffe et al., 2000). However, the ApoE genotype and gender-specific effects, and the precise molecular alterations that cause cognitive decline in AD, have not been systematically investigated at the synaptic termini. Because synapses constitute the fundamental units of information processing in the brain, synaptic dysfunction is believed to be an underlying mechanism for neurodegenerative diseases such as AD. To study synapse in vitro, they are purified into ‘synaptosomes’, after the mild disruption of brain tissue. This tissue disruption causes nerve terminals to detach from their axons, and the post-synaptic and glial cells to which they were connected, and then reseal to form synaptosomes (Whittaker et al., 1964; Schrimpf et al., 2005; Bayes and Grant, 2009). Previous studies have used this system to identify impaired respiratory capacity in cortical synaptosomes from superoxide dismutase 2(SOD-2) null mice (Flynn et al., 2011). Another study investigated the repair of oxidative DNA base damage in mouse synaptosomes during normal aging and in an AD model (Gredilla et al., 2012).

In this current study, we first examined the specific effects of the ApoE genotype and gender on the synaptic proteome in the mouse brain, using label-free quantitative proteomics. Second, we determined the level of oxidative stress in the cortex of human-ApoE transgenic mice, to confirm if the ApoE4 and female gender risk factor induced changes in protein expression had functional consequences. Third, we measured oestrogen levels and aromatase mRNA expression in the cortex of ApoE transgenic mice to further explore the relationship between oestrogen and the ApoE genotype. The protective effect of oestrogen was also assessed in C6 cell stable transfected with different ApoE constructs. Finally, we performed the proteomic profiling in isolated non-synaptic mitochondria in a brain area that was distant from synaptic terminals to assess the effects of ApoE4 genotype and gender.

Method

A brief description of the materials and methods is presented in this section. For a full and detailed description, please refer to the supplementary material.

Animals and treatment

Transgenic mice (Jackson Laboratory, USA) express human ApoE under the control of the human glial fibrillary acidic protein (GFAP) promoter and do not express endogenous mouse ApoE. Mice were maintained on a 12-h light/dark cycle (lights on at 07:00 h), at a temperature of 22±1 °C. Food and water were available ad libitum. All procedures were performed according to the Animal Care and Use Committee of University of Science and Technology of China.

Isolation of synaptosomal and non-synaptic mitochondrial fractions

Synaptosomes and non-synaptic mitochondria were isolated using the Percoll (GE Healthcare, Sweden) gradient method with slight modifications (Dunkley et al., 2008; Zhang et al., 2011).

Cryo-electron tomography (Cryo-ET) of the synaptosomes

Synaptosomes embedded in sucrose solution were stored in –80 °C before use. Sucrose was removed by washing with HEPES-buffered saline (HBS) followed by centrifugation for 15 min at 18000g and the pellets were resuspended in HBS. Four microlitres of sample was mixed with 15 nm gold beads, and added to holey carbon-coated copper grids (Quantifoil Micro Tools, Germany). Extra liquid was absorbed using filter paper, and the grids were then plunged into liquid ethane for rapid vitrification using an FEI Vitrobot (FEI Company, USA).

Data were collected using a Tecnai F20 transmission electron microscope equipped with a FEG and a 4k ×4k CCD camera. The tilt series were aligned and reconstructed using IMOD software (Kremer et al., 1996), and then visualized and segmented with Amira software (TGS, USA).

Trypsin digestion

Fifty microgram protein lysates from the synaptosomes and 20 μg from the non-synaptic mitochondria of each mouse cortex were used. The protein concentration was adjusted to 0.5 μg/μl, and 100 mM DTT was added to a final concentration of 20 mM. All samples were incubated at 37 °C for 2 h, and then 200 mM IAA was added to a final concentration of 40 mM. All samples were incubated for an additional 40 min at room temperature in the dark. Sample were then precipitated with UPPA reagent (Pierce, USA) and centrifuged at 13000g for 20 min at 4 °C. The pellets were solubilized in 100 mM NH4HCO3, pH 8.5 and incubated with sequencing-grade trypsin 1:50, w/w, (Promega, USA) for 16 h at 37 °C in a shaking incubator.
Mass spectrometry (MS)

For RP-LC MS/MS analysis, 500 and 200 ng protein digests from the synaptosomes and non-synaptic mitochondria were re-dissolved in 0.1% formic acid (FA) in water and loaded onto a Biobasic C18 Picofrit™ column (100 mm length, 75 μm ID) (New Objective, USA). Buffer A (0.1% FA in H₂O) and B (0.1% FA in 100% acetonitrile) were used to generate a gradient from 2 to 98% B in 150 min and were introduced into a linear trap quadrupole (LTQ) ion trap mass spectrometer (Thermo Finnigan, USA) using electrospray ionization from a nanoflow probe at 600 nl/min. The LTQ was set as a full MS scan (m/z 300–2000) followed by four consecutive MS/MS scans of the four most intense ions from the MS spectrum. The electrospray voltage and capillary temperature were 1.8 kV and 200 °C, respectively. To prevent repetitive analysis, dynamic exclusion was enabled with a repeat count of one, a repeat duration of 30 s, and exclusion duration of 60 s. Two blank injections were performed before and after sample analysis to minimize carry-over.

Western blot analysis

Cortex tissues were homogenized in Trizol reagent (Invitrogen, USA) containing cocktail. Proteins were separated on 12% SDS-PAGE gels and then transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk solution and probed with antibodies against cytochrome c oxidase (COX) IV (1:2000, Abcam, USA), SOD2 (1:3000, Abcam, USA), PSD95 (1:1500, Abcam, USA), SYN1 (1:3000, Sigma-Aldrich, USA) and β-actin (1:3000, Kangcheng, China). The bands were visualized on X-ray films using ECL reagents (Pierce, USA).

Quantitative determination of glutathione (GSH), glutathione disulfide (GSSG) and malondialdehyde (MDA) levels

GSH and GSSG levels were determined using a protocol described by Rahman et al. (2006). The MDA content in supernatants was determined using kits (Jiancheng, China) and the absorbance was read at 532 nm. All results were expressed as nmol/mg protein.

Oestrogen measurement and aromatase mRNA expression

Mouse 17β-oestradiol (E₂) levels were detected using an enzyme-linked immunosorbent assay kit (Biosource, USA), and the E₂ concentration in the cortex was normalized to total protein. The expression of the ApoE mice aromatase gene was calculated using the 2−ΔΔCt method normalized to actin. The efficiency of the primers was assessed before RT-qPCR was performed.

Results

Isolation and cryo-ET imaging of synaptosomes

Synaptosomes have been exhaustively characterized in functional terms, such as the production of ATP and the
release of neurotransmitters (Dunkley et al., 2008). Synaptosomes from the ApoE transgenic mouse cortex were isolated using the Percoll gradient method (Supplementary Figure S1a). The enrichment and purity of synaptosomes and non-synaptic mitochondria were confirmed by Western blotting (Supplementary Figure S1b). The sub-cellular distribution of synaptosomes was also assessed using the cryo-ET. A 1.5-nm thick virtual section derived from electron tomographic reconstruction displays revealed that the synaptosome, was readily visible, including the structure of nerve terminals, with post-synaptic density, synaptic vesicle (SVs), mitochondrion and synaptic adhesion molecules (Fig. 1a). Furthermore, three-dimensional rendering of the same tomogram clearly resolved the basic spatial configuration of the synaptosome (Fig. 1b).

**ApoE genotype- and gender-dependent alterations in glucose, fatty acids, amino acids and oxidative phosphorylation pathways in the synaptosomal proteome**

Enrichment analyses of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways (Fig. 2a) and Gene Ontology (GO) biological processes (Fig. 2b) showed that the differentially expressed proteins induced by the ApoE genotype or gender risk factor were significantly enriched in signalling pathways linked to metabolism, such as glycolysis, the TCA cycle, amino acid metabolism and oxidative phosphorylation. Heat maps display 167 different proteins and their relative abundance side by side, with a focus on proteins related to energy metabolism, oxidative reduction, synaptic transmission and cytoskeleton (Fig. 2c). Interestingly, 81 of the 1043 synaptosomal proteins detected in all 20 mice were significantly affected by ApoE genotype, gender, or both (Table 1, Fig. 2). Of these 81 proteins, 53 (65.4%) were significantly different between ApoE genotypes, 44 (54.3%) differed with genders, and 19 (23.5%) were significantly affected by both variables. However, a significant effect of interaction between these two factors was found in only four proteins (4.9%): synapsin I ($F_{(1,16)} = 4.871, p = 0.042$), synaptophysin ($F_{(1,16)} = 5.128, p = 0.038$), septin 5 ($F_{(1,16)} = 15.351, p = 0.001$), and tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein (YWHAE) ($F_{(1,16)} = 5.359, p = 0.034$). Among the 53 ApoE genotype dependent proteins, 32 (60.4%) were significantly lower and 21 (39.6%) were significantly higher in ApoE4 mice. Of the gender dependent proteins, 43 (97.7%) were significantly lower in female mice; only the expression of glutathione S-transferase Mu 1 (GSTM1) was increased.
Table 1. Quantitative proteomic analysis of the synaptosomal proteins involved in ApoE genotype- and gender-dependent responses

<table>
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<th>IPI number</th>
<th>Protein name</th>
<th>Gene symbol</th>
<th>ApoE genotype</th>
<th>Gender</th>
<th>Interaction</th>
<th>Ratio</th>
<th>ApoE3/ApoE4</th>
<th>Male/female</th>
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<td>IPI00120212</td>
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<td>Ndufs1</td>
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<td>Uqcrcl</td>
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<td>Ubiquinol cytochrome c reductase core protein 2</td>
<td>Uqrc2</td>
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<td>Cyc1</td>
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<td>Cytochrome c oxidase subunit 2</td>
<td>Cox2</td>
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<td>0.042*</td>
<td>0.711</td>
<td>1.57▲</td>
<td>1.54▲</td>
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<td>ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d</td>
<td>Atp5h</td>
<td>0.938</td>
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<td>ATP synthase, H+ transporting mitochondrial F1 complex, beta</td>
<td>Atp5b</td>
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<td>0.978</td>
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<td>ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1</td>
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<td>Atp5o</td>
<td>0.058</td>
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<td>1.55▲</td>
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<td>Gpi1</td>
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<td>Hexokinase 1</td>
<td>Hk1</td>
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<td>Phosphoglycerate kinase 2</td>
<td>Pglk2</td>
<td>0.964</td>
<td>0.037*</td>
<td>0.716</td>
<td>0.99</td>
<td>1.63▲</td>
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<td>IPI00119458</td>
<td>Aldolase C, fructose-bisphosphate</td>
<td>Aldolc</td>
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<td>Citrate synthase</td>
<td>Cs</td>
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<td>Fumarate hydratase 1</td>
<td>Fh1</td>
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<td>2.02▲</td>
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<td>Isocitrate dehydrogenase 3 (NAD+) beta</td>
<td>Idh3b</td>
<td>0.047*</td>
<td>0.054</td>
<td>0.807</td>
<td>1.60▲</td>
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<td>Malate dehydrogenase 2, NAD</td>
<td>Mdhh2</td>
<td>0.004**</td>
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<td>Shpc1</td>
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<td>Heat shock protein 9</td>
<td>Shpg9</td>
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<td>Peroxisiredoxin 5</td>
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<td>0.003**</td>
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<td>Glutathione S-transferase Mu 1</td>
<td>Gstm1</td>
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<td>0.026*</td>
<td>0.399</td>
<td>0.68</td>
<td>0.57▲</td>
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Table 1. (Cont.)

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<th>Protein name</th>
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<th>Ratio</th>
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<td><em>Oxct1</em></td>
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<td>Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)</td>
<td><em>Dbst</em></td>
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<td>0.112</td>
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<td>Microtubule-associated protein 6</td>
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Subsets of proteins with significantly altered expression were then grouped by genotype or gender, with a cut-off value of a 1.5-fold change \((p<0.01)\) (Fig. 3, Table 1). The levels of seven proteins (NADH dehydrogenase 1 alpha sub-complex 9, NADH dehydrogenase flavoprotein 1, ubiquinol-cytochrome c reductase core protein 1, ubiquinol-cytochrome c reductase, Rieske iron-sulphur polypeptide 1, fumarate hydratase 1(FH1), SOD2 and sideroflexin 3) were significantly lower in ApoE4 mice compared with ApoE3 mice \((n=10)\) (Fig. 3a). Interestingly, the expression of seven proteins (septin 7, synaptin 1b, microtubule-associated protein 6, 2’,3’-cyclic nucleotide 3’ phosphodiesterase, myelin basic protein, proteolipid protein 1 and ATase, Na+/K+ transporting, mitochondrial F1 complex, O subunit) was significantly higher in female mice \((n=10)\) (Fig. 3b).

Oxidative phosphorylation and ATP synthesis were two of the most prominently affected pathways that were down-regulated in ApoE4 mice. Twelve proteins involved in complex I, III, IV and V of the electron transport chain (ETC) had significantly lower expression in ApoE4 mice (Supplementary Figure S2a, Table 1). Interestingly, cytochrome c levels were significantly lower in female mice. The ratios (ApoE3/ApoE4 or male/female) of 81 proteins were shown side by side \(R=\text{mean ApoE3/mean ApoE4 or mean male/mean female}\). Proteins with >1.5-fold changes \((p<0.05, \text{2-way ANOVA})\) are marked with triangle symbols.

Increased oxidative stress induced by ApoE4 and female risk factors

Reduced ETC efficiency and increased oxidative stress impairs neuronal function and exacerbates neurodegeneration. Among the proteins involved in oxidative stress pathway identified in our quantitative proteomics analysis, six of seven significant proteins (SOD1, SOD2, HSPD1, HSPE1, HSPA1 and PRDX5) exhibited reduced
expression in ApoE4 or female mice, except for GSTM1 (Table 1). In particular, SOD2 (p=0.001, ratio=1.82) and PRDX5 (p=0.003, ratio=1.57) were significantly lower in ApoE4 and female mice (both n=10). Interestingly, when gender-dependent proteome alterations were assessed, only GSTM1 was significantly up-regulated, and was ∼1.75-fold higher in female than male mice (p=0.026, ratio=0.57).

COXIV and SOD2 play an important role in maintaining the redox balance. Therefore, Western blotting validated their expression levels. The levels of COXIV (p=0.018, ratio=1.51) and SOD2 (p=0.002, ratio=1.39) were significantly lower in ApoE4 mice compared with ApoE3 mice (n=10) (Fig. 4a, c). Similarly, the levels of both proteins (COXIV: p=0.023, ratio=1.48; SOD2: p=0.002, ratio=1.40) were significantly lower in female mice compared with male mice (n=10) (Fig. 4b, d).

The GSH/GSSG ratio and MDA levels are considered to be indicators of oxidative stress. Our results demonstrate that GSSG levels were increased significantly in ApoE4 (n=10) compared with ApoE3 animals (n=10) (p=0.005, ratio=0.61) (Fig. 5b). Similarly when ApoE4 females (n=10) and ApoE4 males (n=10) were compared (p=0.003, ratio=0.58), there was also an increase (Fig. 5f). In contrast, there was no significant difference in GSH levels in the mouse cortex between genotypes (Fig. 5a) or genders (Fig. 5e). Furthermore, ApoE4 mice (n=10) had a lower GSH/GSSG ratio than ApoE3 mice (n=10) (p=0.025, ratio=1.50) (Fig. 5c) and a similar result was observed in female mice compared with male mice (n=10) (p=0.027, ratio=1.49) (Fig. 5g).

Consistent with above data, MDA levels were also higher in ApoE4 (n=10) (p<0.001, ratio=0.78) (Fig. 5d) and female mice (n=10) (p=0.012, ratio=0.86) (Fig. 5h).

**Synaptic termini were more susceptible to damage than non-synaptic mitochondria in the soma**

To assess if the ApoE genotype or gender caused detrimental effects in synaptic termini and non-synaptic mitochondrial fractions, the ratio of protein abundance between ApoE genotypes or genders was used as an indicator. We identified 503 proteins after protein rollup in the non-synaptic mitochondria isolated from 20 mice cortices. Of these, 44 mitochondrial-related proteins were detected in both non-synaptic mitochondrial and synaptic neuronal fractions from all 20 samples (Table 2). In the non-synaptic mitochondria, there were no significant proteomic differences in these 44 mitochondrial-related proteins between genotypes, and only 10 proteins were decreased significantly in female, with a mean reduction of ∼1.7-fold (Fig. 6a, c, Table 2). In contrast, 31 of the 44 mitochondrial-related proteins, were decreased significantly, with a mean of ∼1.6-fold, in the synaptosomes in ApoE4 mice. Similarly, 26 proteins were decreased significantly, with a mean of ∼1.5-fold in female (Fig. 6b, d, Table 2).

**Reduced oestrogen levels in female ApoE4 mice cortices and significantly enhanced neuroprotective effects of oestrogen in ApoE3-C6 cells**

To investigate the possible mechanism underlying the gender-related differences in the oxidative stress response to ApoE genotype, the levels of local oestradiol in the cortex of ApoE transgenic mice were measured. The results demonstrated that the oestrogen levels in ApoE4 mice showed a downward trend compared with ApoE3 mice in the cortex, but not in serum (Fig. 7a, p=0.06). Importantly, ApoE4 female mice had significantly...
reduced oestrogen levels compared with ApoE3 female mice (Fig. 7b, \( p<0.05 \)), whereas there was no difference between two ApoE genotypes in the cortices of males. Surprisingly, the expression levels of aromatase, which is a key enzyme during oestrogen synthesis, in the cortices was significantly up-regulated in ApoE4 mice compared with ApoE3 mice, particularly in ApoE4 female mice (Fig. 7c, \( p<0.05 \)).

We next further evaluated whether different ApoE genotypes might affect the protective effects of oestrogen. Oxidative stress was induced in the human ApoE-stable transfected C6 cells by treating with 500 \( \mu \)M H\(_2\)O\(_2\) for 24 h. An MTT assay then revealed that H\(_2\)O\(_2\) exposure was toxic to both ApoE3 and ApoE4 C6 cells. However, ApoE3-C6, but not ApoE4-C6 cells exhibited significantly decreased H\(_2\)O\(_2\) toxicity after treatment with oestrogen. As a rapid compensatory effect, the mRNA levels of COXIV and SOD2 were increased (Fig. 8). Surprisingly, oestrogen significantly down-regulated the expression of COXIV and SOD2 mRNA in ApoE3-C6 compared with ApoE4-C6 cells in response to oxidative stress damage (Fig. 8, \( p<0.05 \)).

**Discussion**

This present study demonstrates that the expression patterns of the synaptosomal proteome in ApoE transgenic mice were significantly affected not only by the ApoE genotype, but also by gender. Furthermore, the greater detrimental effects of the ApoE4 genotype...
in female, compared with male mice are consistent with the epidemiological evidence reporting of ApoE4 and gender as risk factors for the development of AD in humans. These early detrimental effects might accumulate predominantly in the synaptic terminals, but not in the neural soma. Oestrogen levels in females, which were affected by the ApoE4 genotype, might alter the synaptosomal proteome and synaptic...
Table 2. The proteomic response of non-synaptic mitochondria far away from synaptic terminals to the ApoE3/4 genotype or gender factor

<table>
<thead>
<tr>
<th>IPI number</th>
<th>Protein name</th>
<th>Gene symbol</th>
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<th>Ratio (synaptosome)</th>
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oxidative stress leading to the neuronal injury as AD progresses.

Previous studies suggested that astrocyte-secreted, human ApoE4-replacement transgenic mice were useful models of AD (Sun et al., 1998). ApoE, which is mainly secreted by glial cells, plays a key role in the central nervous system (Holtzman et al., 2012). The loss of neurons and synapses in the cerebral cortex are classical pathological features in AD. Synaptosomes (a research model for neuronal and synaptic function) contain the complete synaptic molecular structure and are functional, with the ability to uptake, store, and release neurotransmitters (Whittaker, 1993; Ashton and Ushkaryov, 2005). Synaptosomal proteomics has been used to scrutinize global changes in synaptic proteins and aberrant synapse physiology, which is thought to be the basis of various brain disorders (Li and Jimenez, 2008; Bayes and Grant, 2009). Our results of synaptosomal proteomic profiling revealed that the proteins that were significantly regulated by the ApoE genotype were mainly involved in metabolism and mitochondrial function. Perturbations in the physiological function of mitochondria and increased oxidative stress inevitably disturb neuronal function, which is intimately associated with the development and progression of AD (Moreira et al., 2005; Lin and Beal, 2006). Impairment in the ETC, particularly in COX, was recently linked to the onset of sporadic AD. This is consistent with our previous findings, where the brain protein levels of cytochrome c oxidase subunits were also lower in AD, as assessed by Western blotting (Kish et al., 1999) and microarray analyses (Xu et al., 2006). SOD2 is a key enzyme that is responsible for the mitochondrial detoxification of ROS. A previous report demonstrated that over-expression of SOD2 reduced hippocampal superoxide and prevented memory deficits in an AD mouse model (Massaad et al., 2009). Consistent with those findings, our results demonstrated that SOD2 was significantly down-regulated in ApoE4 mice.

Consistent with many observations in AD patients, our results also showed that ApoE4-associated neuropathology might occur via the disruption of mitochondrially regulated lipid, amino acid, energy and glucose metabolism. For example, dihydrolipoamide S-succinyltransferase, pyruvate dehydrogenase E1 alpha 1, glutaminase, glutamate dehydrogenase 1, 4-aminobutyrate aminotransferase, fumarate hydratase 1 and isocitrate dehydrogenase 3 (NAD+) beta were all decreased significantly by >1.5-fold in ApoE4 compared with ApoE3 mice. The hypothesis that increased oxidative stress caused mitochondrial dysfunction via unknown mechanisms was further supported by the presence of markers of lipid peroxidation and the glutathione system in thirteen-month-old ApoE4 transgenic mice. Mahley et al. (2009) and Chen et al. (2011b) have both reported that the structural differences between ApoE4 and ApoE3, contribute to the regulation of their diverse biological functions.
Gender differences in cognitive function are well documented, in both rodents (Gresack and Frick, 2003) and humans (Astur et al., 2004; Postma et al., 2004). Recent studies have used stereological and correlative light and electron microscopy to reveal that males have a significantly higher synaptic density than females in all cortical layers of the temporal neocortex, (Alonso-Nanclares et al., 2008). Similarly, we found that nearly all proteins from the synaptosomes were down-regulated in the female mouse proteome. Gender differences in oxidative stress were also clearly observed in our experiments (Figs. 2–5). When the female gender and ApoE4 risk factors were combined, these effects were even more profound. Our proteomic results demonstrate that the relative changes in proteins induced specifically by the female risk factor had a similar trend to those induced by ApoE4 factor, particularly for NADH dehydrogenase (ubiquinone) flavoprotein 1, cytochrome c oxidase subunit 2, glutamate dehydrogenase 1, VDAC2 and prohibitin 2 (*p<0.05, **p<0.01, ***p<0.001). Surprisingly, however, of the 44 proteins that were significantly altered by the female-specific risk factor, only GSTM1 was up-regulated (*p<0.05, ratio>1.5). Similarly to its role in maintaining the redox balance, GSTM1 might also play an important role in oxidative stress. The proteome profiling of non-synaptic mitochondria far away from synaptic terminals. (a) The genotype- and gender-dependent effects on mitochondrial-related protein expression are shown in non-synaptic mitochondria (b) Similarly, the genotype- and gender-dependent effects on mitochondrial-related protein expression are shown in synaptosomes. The log_{2} ratios (ApoE4/ApoE3 or female/male) of 44 mitochondrial-related proteins identified in both non-synaptic mitochondrial and synaptosomal fractions from all twenty samples are shown side by side [M=log_{2} (mean ApoE4/mean ApoE3) or M=log_{2} (mean female/ mean male)]. Red and green colours represent increased or decreased fold changes, respectively. (c, d) Representative protein expression patterns affected by the genotype or gender factor in the non-synaptic mitochondria and synaptosomes are shown respectively. The protein levels in ApoE4 or female mice were normalized to the mean of protein content in the corresponding control group. The dashed line indicates the relative protein level of controls (ApoE3 or male group). (Values are presented as mean±s.e.m.; n=10 per genotype or gender; 2-way ANOVA, *p<0.05, **p<0.01, ***p<0.001).
role in SOD2 null mice (Hinerfeld et al., 2004). Consistent with our findings, a previous study reported that female mice were more susceptible to ApoE4-induced cognitive impairment than male mice (Raber et al., 2000). These findings might provide a reasonable explanation as to why elderly females, particularly those carrying the ApoE ε4 allele, have a higher risk of developing AD than males (Payami et al., 1996).

Generally, sex hormones play a dominant role in modulating gender-based physiological differences. Previous studies reported that the decrease in oestrogen in females after menopause is a likely risk factor for AD (Wickelgren, 1997; Yue et al., 2005), and that oestrogen might also play an important role in oxidative stress. Interestingly, our data demonstrated that oestrogen levels could be modulated by the ApoE genotype in the cortex, but not in the serum. ApoE4 could affect oestrogen levels by regulating the hormone metabolism in brain. The compensatory increase in aromatase mRNA, which was affected by the ApoE4 genotype might be a possible explanation for the reduced oestrogen levels. However, the activity of aromatase might be another important factor that affects the oestrogen expression. Our proteomic data also showed that the expression of hydroxysteroid (17-beta) dehydrogenase 10, which is essential for maintaining the appropriate levels of sex hormones (Yang et al., 2005), was down-regulated by both ApoE4 genotype and female gender. A substantial and growing body of literature suggests that oestrogens exert their potent neuroprotective effects via a direct or indirect mitochondrial mechanism (Singh et al., 2006; Brinton, 2008b). Recently, a proteomic study suggested that 17β-oestradiol(E2) regulated mitochondrial function, and specifically increased key elements in the TCA cycle, pyruvate metabolism, oxidative phosphorylation, respiratory efficiency and ATP generation, while reducing free radical leakage and oxidative damage (Nilsen et al., 2007). In contrast, a lack of protection from oestrogen increased mitochondrial ROS production (Numakawa et al., 2011) and the dysregulation of Ca2+ homeostasis (Brinton, 2008a). This might cause synaptic dysfunction, leading to neuronal injury in the early stages of AD. In the current study, the protective effects of oestrogen were only found in ApoE3 cells. Consistent with this, the ApoE4 genotype reduced the neuroprotective effect of E2 in an animal model (Brown et al., 2008) Although its mechanism of action remains unclear, ApoE4 was reported to have isoform specific effects on signal transduction pathways including ERK and JNK (Ohkubo et al., 2001; Korwek et al., 2009). In addition, oestrogen-mediated signalling involved activation...
of the ERK and JNK pathways (Cui et al., 2013). Therefore, it is possible that oestrogen signalling is defective in the ApoE4 genotype. Furthermore, the results of our study might explain why oestrogen replacement therapy was associated with reduced cognitive decline among ApoE4-negative females (Yaffe et al., 2000).

Our proteomic analyses indicated that synaptic terminals were more susceptible to oxidative stress damage that was affected by the ApoE genotype and gender factors. In synaptic mitochondria, 72.7 and 59% proteins were significantly affected by the ApoE genotype and gender factors, respectively (Table 2, Fig. 6). However, only 22.7% proteins were significantly affected by gender, and none were significantly regulated by ApoE genotype in non-synaptic mitochondria (Table 2, Fig. 6). The expression levels of voltage-dependent anion-selective channel protein 2 (VDAC2) and prohibitin 2, which are located in the outer and inner mitochondrial membranes respectively, were comparable in the non-synaptic mitochondria from either genotype or gender. In contrast, the levels of these two proteins were significantly down-regulated in ApoE4 and female mice in the synaptosomes. The differential expression of PRDX5 (an antioxidant enzyme), which was affected by ApoE4 genotype and female gender, also has a similar synaptic regional specificity. These findings are interesting and the down-regulation of these evolutionarily conserved and multifunctional proteins could induce changes in mitochondrial function, leading to excessive ROS production. Recently, many studies have supported the notion that prohibitins participate in mitochondrial dynamics and stabilize the oxidative phosphorylation system (Artal-Sanz and Tavernarakis, 2009). The deletion of VDAC2 enhanced the activation of mitochondrial apoptosis (Cheng et al., 2003). PRDX5 confers protection against oxidative stress and promotes longevity in Drosophila melanogaster (Radyuk et al., 2009). Consistent with our findings, recent studies also reported the regional specificity of the mitochondria in neurons, and also proposed that synaptic mitochondria are long-lived, but more vulnerable to cumulative oxidative stress than non-synaptic mitochondria. In addition, synaptic mitochondria were more susceptible to Aβ-induced damage than mitochondria located in other regions in an AD mouse model (Du et al., 2010). Other reports also demonstrated that synaptic mitochondria might initiate neuronal death in response to insults, increasing the synaptic levels of intracellular Ca2+ (Brown et al., 2006). Therefore, significant evidence suggests that defects in synaptic mitochondria can likely lead to synaptic dysfunction and the loss of the synapses, which lead to alterations in cognitive function during AD.

Our findings revealed that the abnormal metabolism in the synapse and increased oxidative stress might represent a functional convergence of the ApoE4 and gender risk factors. Furthermore, the synaptic terminal is more vulnerable to cumulative oxidative stress damage induced by the ApoE4 genotype or female gender. The decreased oestrogen levels in the cortex observed with the ApoE4 genotype might limit the neuroprotective effects of oestrogen against oxidative stress, which leads to synaptic dysfunction and further neuronal injury.

Supplementary material
For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000601

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Statement of Interest
None.

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