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Association between CAG repeat length in the *PPP2R2B* gene and Alzheimer disease in the Japanese population

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ABSTRACT

We analyzed the association between *PPP2R2B* gene CAG repeat length and Alzheimer disease (AD) susceptibility in the Japanese population. Blood samples were collected from 218 late-onset AD patients and 86 controls. DNA fragments containing the target CAG repeat region were amplified using polymerase chain reaction (PCR). PCR products were sequenced using ABI PRISM 310 genetic analyzer. The mean CAG repeat length did not differ significantly between the control and AD groups. In contrast, the frequency of CAG repeats shorter than 15 was significantly higher in AD group, specifically in the AD with APOE4 subgroup, than in the control group. The results suggest that CAG repeat lengths in the *PPP2R2B* gene may be potential genetic markers for AD susceptibility in the Japanese population.

Alzheimer disease (AD) is the most common cause of dementia in the elderly, and is characterized by progressive cognitive decline and cerebral atrophy. The primary pathological feature of AD is the presence of neurofibrillary tangles and senile plaques in the brain [26]. The presence of the ε4 allele of the apolipoprotein E (APOE) gene (*APOE4*) confers a heightened risk of late-onset AD in multiple genetic backgrounds [4]. Although trinucleotide repeats are common features of the human genome, the trinucleotide repeat number varies among individuals and the lengths of these repeats is associated with many genetic diseases, including Huntington disease (HD) and Dentatorubral-pallidoluysian atrophy (DRPLA) [25]. A majority of spinocerebellar ataxias (SCAs) are caused by the expansion of trinucleotide repeats. SCAs are a group of autosomal dominant progressive neurodegenerative disorders that are characterized by overlapping and variable phenotypes [20]. Spinocerebellar ataxia type 12 (SCA12) is caused by CAG repeat expansion in the non-coding region of the *PPP2R2B* gene [11]. Clinical symptoms of SCA12 include dementia, upper limb tremor, and extra pyramidal symptoms. Brain magnetic resonance images of the affected individuals revealed cerebral and cerebellar atrophy [11,23].

The *PPP2R2B* gene, which encodes a brain-specific regulatory B subunit of the serine/threonine protein phosphatase 2A (PP2A), is located on chromosome 5q31–33 and is widely expressed in brain neurons [21]. PP2A has been implicated in cell cycle and proliferation and development and regulation of multiple signal transduction pathways [30]. In addition, PP2A dephosphorylates the hyperphosphorylated tau protein [7]. It is suggested that PP2A-mediated dephosphorylation of tau is facilitated by the B regulatory subunit of PP2A [6]. Tau, an axonal microtubule-associated protein, promotes microtubule assembly and stabilization [17], and tau phosphorylation has been implicated, to varying degrees, in AD pathogenesis [12]. Because of the overlap between the SCA12 phenotype and certain aspects of AD, including the functional role of PP2A, it is important to determine the association between the *PPP2R2B* gene and AD. Recently, Chen et al. reported that the presence of short alleles of the CAG repeat in the *PPP2R2B* gene is associated with increased AD susceptibility in the Han Chinese [3]. However, the existence of such an association among other population group is uncertain. In the present study, we investigated the association between *PPP2R2B* gene CAG repeat lengths and AD susceptibility in the Japanese population.

Patients with late-onset AD were diagnosed with definite or probable AD according to the criteria of the National Institute of Neurological and Communicative Disorders and Stroke Alzheimer's Disease and Related Disorders Association [22]. The control group consisted of non-demented elderly subjects from the general population. After written informed consent was obtained, peripheral blood was collected from 218 late-onset AD patients (mean age: 79.0 years; women: 65.6%) and 86 control subjects (mean age: 74.7 years; women: 52.3%). The protocol for specimen collection was approved by the Genome Ethical Committee of Osaka University Graduate School of Medicine.

DNA was extracted from peripheral blood nuclear cells using the phenol–chloroform method or the QIAamp DNA Blood Kit (Qiagen). CAG repeats in the *PPP2R2B* gene were identified

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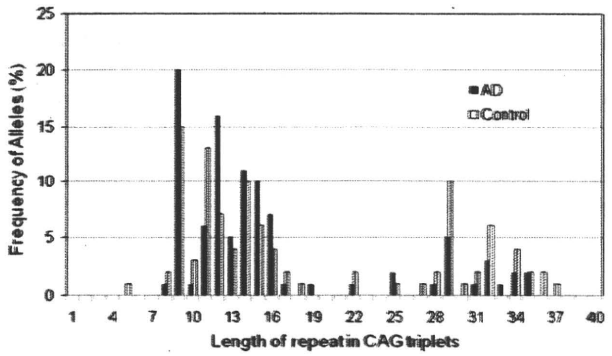

Fig. 1. Distribution of allele frequencies against the CAG repeat numbers in the *PPP2R2B* gene of control subjects and AD patients.

by polymerase chain reaction (PCR) amplification using 6FAM dye-labeled forward (5′-TGCTGGGAAAGAGTCGTG-3′) and reverse (5′-GCCCGCGCACTCACCCTC-3′) primers. The PCR was performed with 36 cycles consisting of two cycles of 30 s at 95 °C and 30 s at 70 °C, two cycles of 30 s at 95 °C and 30 s at 65 °C, two cycles of 30 s at 95 °C and 30 s at 60 °C, and 30 cycles of 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C preceded by 10 min at 95 °C and followed by 10 min at 72 °C. PCR products were electrophoresed in a capillary in an automated ABI PRISM 310 genetic analyzer (Applied Biosystems). Analysis was performed with GenScan analysis software (Applied Biosystems) **[11]**. The *APOE* genotype was determined using a PCR-RFLP method **[15]**.

Statistical analysis was performed using JMP (version 7.0, SAS Institute, Cary, NC). The 2-sided Mann–Whitney's *U*-test was used to evaluate the difference in CAG repeat distribution between the AD and control groups. The difference in the CAG repeat allele frequencies between the groups was further tested by the Chi-square test. Each value represents mean (standard error). A *p*-value of <0.05 was considered statistically significant.

The frequency distribution of CAG repeat alleles in the *PPP2R2B* genes was analyzed in 218 LOAD patients and 86 controls. In **Fig. 1**, the CAG repeat number (*X*-axis) is plotted against the frequency of distributions (%) (*Y*-axis). The repeat range was 5–37 and 8–35 in the control and AD groups, respectively. Pathological expansion of CAG repeats was not detected in the AD and control groups. The most common lengths were 9 (15.3%) triplets in the control group. Similarly, in the AD group, the most common lengths were 9 (20.0%) triplets. The mean CAG repeat lengths in the AD and control groups (14.2 and 16.6, respectively) were not statistically different ($p = 0.158$). In addition, when we divided the AD group into APOE4 and non-APOE4 subgroups, we found that the mean CAG repeat lengths of both subgroups (13.9 and 14.5, respectively) were not significantly different from that of the control group (**Table 1**).

Table 2
Short (≤15) and long (15>) alleles: CAG repeat number in *PPP2R2B*; the short and long allele repeat numbers in the AD and control groups were compared.

Group	Allele number			*p* value	OR
	Total	Short (≤15)	Long (>15)		
Control	172	110 (64%)	62 (36%)		
Control with APOE4	24	16 (67%)	8 (33%)	0.267	
Control without APOE4	148	94 (64%)	54 (36%)	0.022*	1.58
AD	436	320 (73%)	116 (27%)	0.021*	1.55
AD with APOE4	212	163 (77%)	49 (23%)	0.005*	1.87
AD without APOE4	224	157 (70%)	67 (30%)	0.197	

Differences in the allele repeat numbers in the AD and control groups were determined using Chi-square test.
* $p < 0.05$, statistically significant.
OR, odds ratio.

Because the mean CAG repeat length among all subjects was 15, we dichotomized the alleles into short (≤15) and long (15>) categories. Statistical analysis revealed that the frequency of CAG repeats shorter than 15 was significantly higher in the AD group than in the control group ($p = 0.021$, odds ratio = 1.55) (**Table 2**). Compared to the controls, the AD subgroups, APOE4 and non-APOE4, each had a significantly higher frequency of CAG repeats shorter than 15 ($p = 0.005$, odds ratio = 1.87). However, there was no significant difference in the allele frequency distribution between the non-APOE4 AD group and the control group ($p = 0.197$) (**Table 2**). Additionally, a comparison of the allele frequency distributions of the control subgroups, APOE4 and non-APOE4 with that of the AD revealed that the frequency of CAG repeats shorter than 15 was significantly higher in the AD groups than in the control without APOE4 groups ($p = 0.022$, odds ratio = 1.58) (**Table 2**).

SCA12 is a relatively rare late-onset neurodegenerative disorder characterized by diffuse cerebral and cerebellar atrophy **[11]**. The phenotype typically involves action tremor of upper extremities and various symptoms, including dementia. SCA12 is caused by CAG repeat expansion in the non-coding region of the *PPP2R2B* gene **[10,11]**. Pathogenic CAG repeat expansions have been detected in SCA12 patients in the range of 55–69 to 66–78, but normal individuals from different ethnic populations have exhibited ranges from 7–28 to 9–45 **[2,3,5,11,27–29]**. A correlation between the SCA12 phenotype and certain aspects of AD has been suggested. However, the lone study that analyzed the association between CAG repeat expansions in the *PPP2R2B* gene and AD susceptibility reported that the frequency of the Han Chinese individuals carrying the short 5-, 6-, and 7-triplet alleles was notably higher in AD patients **[3]**.

In the present study, we investigated the length of *PPP2R2B* gene CAG repeats in AD patients and control subjects in the Japanese population. The mean CAG repeat lengths in the AD and control groups were not statistically different. In contrast, we found that the frequency of CAG repeats shorter than 15 was significantly higher in the AD group, specifically the AD with APOE4 subgroup

Table 1
Comparison of CAG repeat numbers in control subjects and AD patients.

Group	Control			AD		
	Total	APOE4 (+)	APOE4 (−)	Total	APOE4 (+)	APOE4 (−)
Number	86	12	74	218	106	112
Allele range	5–37	9–34	5–37	8–35	8–35	8–35
Allele with maximum frequency						
Allele	9	9	9	9	9	9
Frequency (%)	15.3	14.2	16.7	20.0	20.1	17.5
Mean (SE)	16.6 (0.8)	14.4 (1.8)	16.9 (0.8)	14.2 (0.5)	13.9 (0.6)	14.5 (0.7)
p value		0.942	0.114	0.158	0.110	0.362

The differences between the CAG repeat numbers in the control and AD groups were assayed using Mann–Whitney's *U*-test.
SE: standard error of the mean.

than in the control group (**Table 2**). Our results suggested that AD is associated with a lower number of CAG repeats in the *PPP2R2B* gene. This was similar to the findings of a previous report by Chen et al. **[3]**. However, in our AD patients, we did not find short 5–7 triplet alleles which detected in AD patients in the Han Chinese population. This discrepancy may reflect a genetic differentiation between the Han Chinese and Japanese populations.

The presence of the ε4 allele of *APOE* gene confers a heightened risk of late-onset AD **[4]**. As compared to individuals without the ε4 alleles, the risk for AD is 2- to 3-fold and about 12-fold higher in individuals carrying one and two ε4 alleles, respectively **[1,14,24]**. Though several studies have attempted to elucidate the mechanism for this increased risk, how APOE4 influences AD progression has yet to be proven. In particular, we found that the frequency of short CAG repeats (≤15) was higher in the AD with APOE4 group than in the control group. Therefore, it is likely that a short number of CAG repeats of *PPP2R2B* gene play an important role for the progression of late-onset AD with APOE4.

PP2A is composed of three subunits: a catalytic subunit (C), a scaffolding subunit (A), and a regulatory subunit (B). Assembly of the complex with the regulatory B subunit is required for the specificity and regulation of PP2A **[31]**. In addition, PP2A is the major tau phosphatase that dephosphorylates tau at multiple sites, and its activity is decreased by 30% in the frontal or temporal cortex of AD patients compared to controls **[8,18]**. This down-regulation of PP2A activity in AD brains is thought to be partially responsible for abnormal tau phosphorylation. Therefore, differences in the CAG repeat lengths in the *PPP2R2B* gene may regulate PP2A activity, leading to AD progression. Through a reporter assay, the short 5–7 triplet alleles were shown to be associated with decreased PPP2R2B promoter activities **[3]**. However, it has not yet been demonstrated that the short CAG repeat lengths in the PPP2R2B affect PP2A function directly.

APOE plays an important role in the distribution and metabolism of cholesterol in the human body **[19]**. APOE4 has also been associated with tau hyperphosphorylation in several animal models **[9]**. In particular, high cholesterol such as in Niemann-Pick C disease might be involved in decreasing membrane fluidity **[16]**. Therefore, it was recently supposed that signal transduction through the interaction of APOE4 with the neuronal cell membrane might involve AD progression through various kinases and phosphatases **[13]**.

In conclusion, our results suggest that CAG repeat lengths in the *PPP2R2B* gene may be potential genetic markers for AD susceptibility in the Japanese population. Further investigations are required to confirm the role of the *PPP2R2B* gene in AD using a larger sample size and a different population group.

Conflicts of interest

None of the authors has any conflicts of interest.

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Restraint-Induced Expression of Endoplasmic Reticulum Stress-Related Genes in the Mouse Brain

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ABSTRACT

Depression is a significant public health concern but its pathology remains unclear. Previously, increases in an endoplasmic reticulum (ER) stress-related protein were reported in the temporal cortex of subjects with major depressive disorder who had died by suicide. This finding suggests an association between depression and ER stress. The present study was designed to investigate whether acute stress could affect the ER stress response. Mice were immobilized for a period of 6 hr and then expression of ER stress response-related genes was measured by real-time PCR. We also used enzyme-linked immunosorbent assay for concomitant measurement of the plasma corticosterone levels in the mice. The effect of corticosterone on ER stress proteins was further investigated by treating mice with corticosterone for 2 weeks and then measuring ER protein expression by Western blotting. After a 6 hr restraint stress, mRNA levels of ER stress-related genes, such as the 78-kilodalton glucose regulated protein (GRP78), the 94-kilodalton glucose regulated protein (GRP94), and calreticulin, were increased in the cortex, hippocampus, and striatum of mouse brain. Blood plasma corticosterone level was also increased. In the corticosterone-treated mouse model, the expression of GRP78 and GRP94 was significantly increased in the hippocampus. These results suggest that acute stress may affect ER function and that ER stress may be involved in the pathogenesis of restraint stress, including the development of depression.

Keywords: *Corticosterone, Depression, Endoplasmic Reticulum Stress, Restraint Stress*

1. Introduction

Major depression, along with bipolar disorder, has become a common psychiatric disorder in modern society. About 1% of the population is estimated to be affected by major depression one or more times during their lifetime [1]. Even though extensive studies have led to a variety of hypotheses regarding the molecular mechanism underlying depression, the pathogenesis of this disorder remains to be fully elucidated.

The endoplasmic reticulum (ER) is the cell organelle where secretory and membrane proteins are synthesized and folded. It also functions as a Ca^{2+} store and resource of calcium signals. The disturbance of ER functions through events such as disruption of Ca^{2+} homeostasis, inhibition of protein glycosylation or disulfide bond formation, hypoxia and viral or bacterial infection, can result in the accumulation of unfolded or misfolded proteins and may trigger stress responses in the cell (ER stress). To overcome ER stress, an unfolded protein response (UPR) is invoked by the activation of several signaling pathways; this UPR promotes an adaptive response to ER stress and reestablishes homeostasis in the ER [2,3]. Molecular chaperones such as the 78-kilodalton glucose regulated protein (GRP78) and the 94-kilodalton glucose regulated protein are induced and promote correct protein folding. If the damage is too severe to repair, C/EBP-homologous protein (CHOP) and other factors are activated and induce cell apoptosis [4]. On the other hand, if misfolded protein aggregates into insoluble higher-order structures, it can give rise to various diseases. For example, rhodopsin misfolding causes autosomal dominant retinitis pigmentosa [5], while the accumulation of amyloid β-peptide is associated with Alzheimer's disease [6].

Some reports have also suggested a relationship between mental disorder and ER stress. In bipolar disorder patients, DNA microarray analysis of cell derived from twins discordant with respect to the disease revealed a down-regulated expression of genes related to ER stress responses such as x-box binding protein 1 (XBP1) and GRP78 [7]. In schizophrenia patients, a similar abnormality of these genes was found [8]. In addition, mood-stabilizing drugs such as valproate and lithium have been reported to increase the expression of GRP78, GRP94, and calreticulin [9]. Similarly, olanzapine, one of the second-generation "atypical" anti-psychotic drugs, appears to potentiates neuronal survival and neural stem cell differentiation by regulation of ER stress response proteins [10].

A recent study reported that significantly increased levels of GRP78, GRP94, and calreticulin were found in the temporal cortex of subjects with major depressive disorder who had died by suicide compared with control subjects who had died of other causes [11]. In addition, hippocampal atrophy [12] and reduction of glial density in the subgenual prefrontal cortex [13] were found in patients with major depression. Stress, a risk factor for depression, has been shown to induce atrophy of the apical dendrites of the hippocampal neurons [14], and to promote neuronal apoptosis in the cerebral cortex [15] in animal depression models. These findings suggest that a stressful situation, which may increase the risk for suicide, serves as an ER stressor. To clarify the relationship between exogenous stress and ER stress, in the present study, we investigated the expression of ER stress-related genes after restraint stress. We also focused on the elevation of corticosterone in the plasma and used a corticosterone-treated depression model to clarify the relationship between chronic corticosterone elevation and ER stress.

2. Materials and Methods

2.1. Animals

Male 9-week-old ddY mice and male 6-week-old ICR mice (Japan SLC, Hamamatsu, Japan) were used for all experiments. Mice were housed at 24 ± 2°C under a 12 hr light-dark cycle (lights on from 8:00 to 20:00) and had ad libitum access to food and water when not under restraint. Animals were acclimatized to laboratory conditions before the experiment. All procedures relating to animal care and treatment conformed to the animal care guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University. All efforts were made to minimize both suffering and the number of animal used.

2.2. Restraint Stress

Male 9-week-old ddY mice (Japan SLC) weighing 30-40 g were used for real-time PCR studies. Mice were placed into 50-mL perforated plastic tubes, which prevented them from turning in any direction. Each mouse was maintained in the tube for 6 hr without any access to food or water.

2.3. Sampling

After this restraint stress, a blood sample was collected from the tail and the mouse was decapitated. The brain was quickly removed from the skull, briefly washed in ice-cold saline, and laid on a cooled (4°C) metal plate. The brain was rapidly dissected to separate the hippocampus, striatum, and cortex and stored at -80°C until use.

2.4. RNA Isolation

Total RNA was isolated from frozen brain using High Pure RNA Isolation Kit (Roche, Tokyo, Japan). RNA concentrations were determined spectrophotometrically at 260 nm. First-standed cDNA was synthesized in a 20-μl reaction volum using a random primer (Takara, Shiga, Japan) and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

2.5. Reai-Time PCR

Real-time PCR (TaqMan; Applied Biosystems, Foster City, CA, USA) was performed as described previously [16]. Single-standard cDNA was synthesized from total RNA using a high capacity cDNA archive kit (Applied Biosystems). Quantitative real-time PCR was performed using a sequence detection system (ABI PRISM 7900HT; Applied Biosystems) with a PCR master mix (TaqMan Universal PCR Master Mix; Applied Biosystems), according to the manufacturer's protocol. A gene expression product (Assays-on-Demand Gene Expression Product; Applied Biosystems) was used for measurements of mRNA expression by real-time PCR. The primers used for amplification were as follows: GRP78: 5'-GTTTG CTGAGGAAGACAAAAAGCTC-3' and 5'-CACTTCC ATAGAGTTTGCTGATAATTG-3'; CHOP: 5'-GGAG CTGGAAGCCTGGTATGAGG-3' and 5'-TCCCTGGT CAGGCGCTCGATTTCC-3'; GRP94: 5'-CTCACCATT TGGATCCTGTGTG-3' and 5'-CACATGACAAGATT TTACATCAAGA-3'; calreticulin: 5'-GCCAAGGACG AGCTGTAGAGAG-3' and 5'-GGTGAGGGCTGAAG GAGAATC-3'; ERdj4: 5'-TCTAGAATGGCTACTCCC CAGTCAATTTTC-3' and 5'-TCTAGACTACTGTCCT GAACAGTCAGTG-3'; EDEM: 5'-TGGGTTGGAAAG CAGAGTGGC-3' and 5'-TCCATTCCTACATGGAGG TAG-3'; p58IPK 5'-GAGGTTTGTGTTTGGGATGCAG-3' and 5'-GCTCTTCAGCTGACTCAATCAG-3'; ASNS: 5'-AGGTTGATGATGCAATGATGG-3' and 5'-TCCC CTATCTACCCACAGTCC-3'; β-actin: 5'-TCCTCCCT

GGAGAAGAGCTAC-3' and 5'-TCCTGCTTGCTGAT CCACAT-3' The thermal cycler conditions were as follows: 2 min at 50°C and then 10 min at 95°C, followed by two-step PCR for 50 cycles consisting of 95°C for 15s followed by 60°C for 1 min. For each PCR measurement, we checked the slope value, R^2 value, and linear range of a standard curve of serial dilutions. All reactions were performed in duplicate. The results were expressed relative to a β-actin internal control.

2.6. Measurement of Plasma Corticosterone

Plasma was obtained as described previously [17] and the concentration of corticosterone was determined *via* a corticosterone EIA kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's protocol.

2.7. Chronic Corticosterone Treatment

Male 6-week-old ICR mice (Japan SLC) weighing 20-25 g were used for chronic oral corticosterone exposure as described in a previous report [18]. Briefly, corticosterone (25 μg/mL free base; 4-pregnen-11β 21-DIOL-3 20-DIONE 21-hemisuccinate; Steraloids, Inc., RI, USA) was add to tap water and the pH was brought to 12-13 with 10 N NaOH (Kishidai Chemical, Osaka, Japan), followed by stirring at 4°C until dissolved (3 to 7 hr). Following dissolution, the pH was brought to 7.0-7.4 with 10 N HCl (Wako, Osaka, Japan). Group-housed ICR mice were presented with this corticosterone solution in place of normal drinking water for 14 days, resulting in a dose of approximately 8.7 mg/kg/day (p.o). Animals were weaned with 3 days of 12.5 μg/mL, and then 3 days with 6.25 μg/mL, to allow for gradual recovery of endogenous corticosterone secretion.

2.8. Western Blot Analysis

At 35 days, each mouse was decapitated and its brain was quickly removed from the skull, briefly washed in ice-cold saline, and laid on a cooled (4°C) metal plate. The brain was rapidly dissected to separate the hippocampus and stored at -80°C until use. Brain samples were homogenized in 10 mL/g tissue ice-cold lysis buffer [50 mM Tris-HCl (pH 8.0) containing 159 mM NaCl, 50 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitor mixture] using a homogenizer (Physcotron; Microtec Co. Ltd., Chiba, Japan). Lysates were centrifuged at 12,000×g for 15 min at 4°C. Supernatants were collected and boiled for 5 min in SDS sample buffer (Wako). Equal amounts of protein were subjected to 10% SDS-PAGE gradient gel and then transferred to poly (vinylidene difluoride) membranes (Immobilon-P; Millipore, MA, USA). After blocking with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with primary antibody. The primary antibodies used were as follows: mouse anti-BiP antibody (BD Bioscience, CA, USA) for GRP78, mouse anti-KDEL antibody (Stressgen Bioreagents Limited Partnership, B.C., Canada) for GRP94, and mouse anti-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). Subsequently, the membrane was incubated with the secondary antibody [goat anti-mouse (Pierce Biotechnology, IL, USA)]. The immunoreactive bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and then measured using LAS-4000 mini (Fujifilm, Tokyo, Japan).

2.9. Statistical Analysis

Statistical comparisons were made by Student's *t*-test using Statview version 5.0 (SAS Institute Inc., NC, USA), with $p < 0.05$ being considered statistically significant.

3. Results and Discussion

Real-time PCR was carried out to investigate whether the expression of ER stress response-related genes in the brain was changed by 6-hr restraint stress. In this study, we investigated the expression of GRP94, carleticulin, ER degradation-enhancing α-mannosidase-like protein (EDEM), protein kinase inhibitor of 58 kDa ($p58^{IPK}$), asparagines synthetase (ASNS), GRP78, ER-localized DnaJ 4 (ERdj4), and C/EBP homologous protein (CHOP). The expression of GRP78, GRP94, and calreticulin mRNA was significantly increased in the hippocampus, striatum, and cortex (**Figure 1**). In addition, there was significantly increased expression of $p58^{IPK}$ mRNA in the cortex, but not in the hippocampus or striatum.

We next investigated whether restraint stress affected the plasma concentrations of corticosterone, as previously reported. Immediately following the 6-hr restraint stress, significantly higher plasma corticosterone concentrations were found in stressed mice compared to unstressed mice. Seven days after the restraint stress, the plasma corticosterone recovered to the normal control level (**Figure 2**).

To clarify the mechanism of ER stress-related mRNA elevation, we artificially elevated the plasma concentrations of corticosterone in mice for 2 weeks and then measured the levels of ER stress-related proteins. In the corticosterone-treated animal model, the expression of GRP78 and GRP94 in the hippocampus was significantly increased compared to control levels (**Figure 3**).

Restraint stress is used widely to induce stress responses in animals, and it is known that a number of stresses, including restraint stress, can cause depression in animals. In the present study, we found that several ER stress-related genes were increased in the mouse hippocampus, striatum, and cortex after restraint stress.

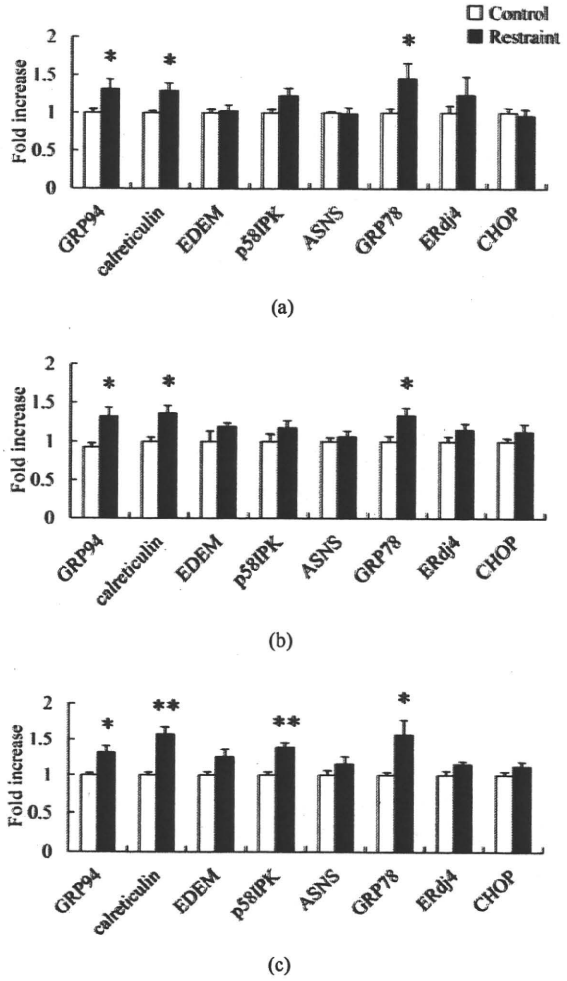

Figure 1. The expression mRNA of ER stress-related factors in the mouse brain after 6 hr restraint-stress. Mice were immobilized for 6 hr in a 50-mL perforated plastic tube. White and black bars represent the control group and the restraint group, respectively. Immediately after restraint, mice were killed and real-time PCR was performed on brain tissues from the (a) hippocampus, (b) striatum, and (c) cortex. Data represent means and S.E.M., n = 3 to 5. *p < 0.05, **p < 0.01 vs. control group. GRP94: the 94-kilodalton glucose regulated protein, EDEM: ER degradation-enhancing α-mannosidase-like protein, p58IPK: protein kinase inhibitor of 58 kilodalton, ASNS: asparagines synthetase, GRP78: the 78-kilodalton glucose regulated protein, ERdj4: ER-localized DnaJ 4, CHOP: C/EBP-homologous protein.

The significant increases in expression of GRP78, GRP94, and calreticulin agreed with the findings of a previous report of changes in the temporal cortex of subjects with major depression who died by suicide [11]. However, no study has yet specifically investigated expression changes of these genes in the hippocampus or the striatum in subjects with depression.

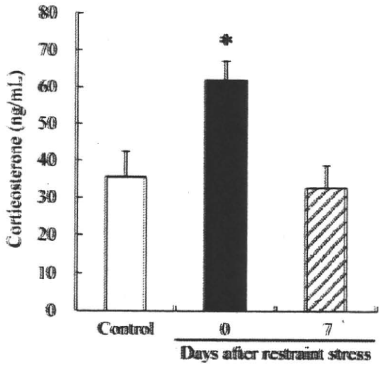

Figure 2. The effect of 6 hr restraint stress on the concentration of corticosterone in mouse plasma. Mice were immobilized for 6 hr. Immediately after restraint and 7 days later, blood samples were collected and concentration of plasma corticosterone was measured by ELISA. Restraint stress significantly increased the concentration of corticosterone in plasma. The corticosterone levels decreased to the normal control levels 7 days after restraint stress. Data represent means and S.E.M., n = 7. *p < 0.05 vs. control group.

GRP78, otherwise known as BiP, is one of the best-characterized ER chaperone proteins and is regarded as a classical marker of UPR activation. Overexpression of GRP78 has been reported to inhibit the upregulation of CHOP, which plays a key role in regulating cell growth and which has been implicated in apoptosis [19,20]. GRP94 and calreticulin are also ER chaperone proteins and show protective effects against ER stress [21]. The increase in these chaperones after restraint stress (**Figure 1**) may represent an attempt to oppose the toxic effect of prolonged stress and the high concentrations of glucocorticoid, such as corticosterone, on the brain. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, which controls glucocorticoid levels, has been reported in most depression patients and glucocorticoid level of depression patients was higher than those of normal ones [22-24]. In the mice in the present study, 6-hr restraint stress elevated the concentration of corticosterone in plasma, suggesting that restraint stress induced a response similar to depression.

Recently, corticosterone has been reported to exert immunostimulatory effects on macrophages *via* induction of ER stress [25]. Following corticosterone treatment, the glucocorticoid receptor (GR) binds onto B-cell lymphoma 2 (Bcl-2), a protein that affects cytochrome C and calcium release from mitochondria. Subsequently, this GR/Bcl-2 complex moves into mitochondria and regulates mitochondrial functions in an inverted "U"-shaped manner–i.e., a high dose treatment with corticosterone decreased levels of GRs and Bcl-2 in mitochondria and intracellular calcium was increased [26,27]. Substances

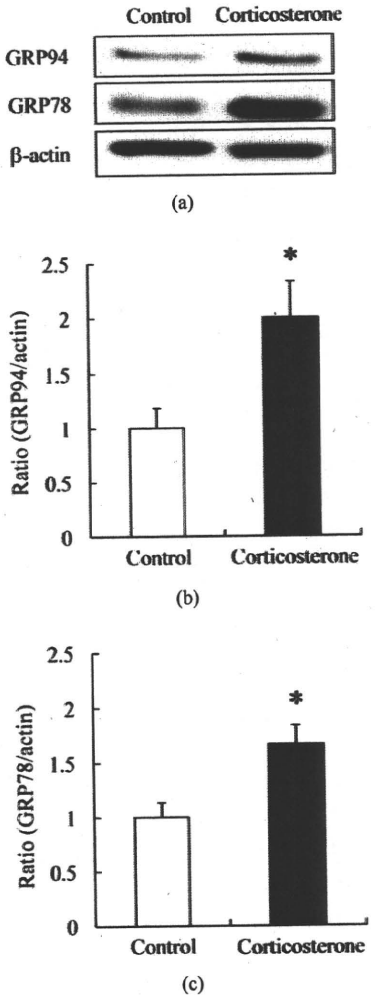

Figure 3. The expression of GRP78 and GRP94 in the hippocampus in a mouse model of chronic corticosterone induced depression. (a) Representative band images show immunoreactivities against GRP94, GRP78, and β-actin. (b) GRP78 expression was significantly increased by corticosterone exposure. (c) GRP94 expression was also increased by corticosterone exposure. Data represent means and S.E.M., n = 5 or 6. *p < 0.05 vs. control group.

that deplete the ER Ca^{2+} stores, such as thapsigargin, are widely used an ER stressors. Therefore, elevation of Ca^{2+} *via* GR may be sufficient for control of ER stress responses. In the present study, the restraint stress induced the expressions of only GRP78, GRP94, and calreticulin, but not other ER proteins. GRP78, GRP94, and calreticulin function as Ca^{2+} binding proteins [28]. Under the high concentration of corticosterone, the intracellular Ca^{2+} level might be higher, therefore, the expressions of GRP78, GRP94, and calreticulin might be increased.

Intracerebroventricular administration of thapsigargin has been reported to produce a depressant-like behavior [29]. A 14-days corticosterone treatment has also shown to induce depression symptoms in mice [18]. We used this animal model to investigate the effect of chronic elevation of corticosterone on ER stress responses in brain. As expected, significant increases in GRP78 and GRP94 proteins were observed in the hippocampus (**Figure 3**). The increase of GRP78 was consistent with the result of a previous report [30]. On the other hand, no change in these proteins was observed in the cortex (data not shown). Mineralocorticoid receptor (MR) and GR, which are the targets of corticosterone, are known to be well expressed in the hippocampus [31,32]. These reports, together with our findings, indicate that the hippocampus may be more sensitive to corticosterone exposure than are other brain regions. Many reports have referred to hippocampal atrophy in patients with depression [12,14]. In the cortex, it had been reported that chronic stress increased the caspase-3 positive neurons, in other words, exogenous stress was contributing to the cell apoptosis [15]. In our study, corticosterone exposure was performed for 2 weeks, but, in fact, long-term cortisol elevation has been observed in most depression patients. More extended corticosterone treatment may affect the expression of ER stress proteins in the cortex.

Recently, many experiments have focused on the relationship between depression and neurogenesis. Interestingly, ER stress also affects adult neurogenesis in the brain [33]. Brain-derived neurotrophic factor (BDNF), which promotes neurogenesis, is also known to inhibit neuronal cell death induced by ER stress [34]. These reports may also point to an involvement of ER stress in depression.

4. Conclusions

Restraint stress, which may contribute to depression in mice, may up-regulate the ER stress response *via* corticosterone elevation. This suggests the possibility of an ER stress involvement in the pathogenesis of stress-related depression disorders.

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Review Article

Apolipoprotein E and central nervous system disorders: Reviews of clinical findings

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Dementia is a major health problem in developed countries with over 25 million people affected worldwide and probably over 75 million people at risk during the next 20 years. Alzheimer's disease (AD) is the most frequent cause of dementia (50–70%), followed by vascular dementia (30–40%), and mixed dementia (15–20%). AD pathogenesis is still to be elucidated but it is believed to be the complex interaction between genetic and environmental factors in later life. Three causative genes for familial AD have been identified: amyloid precursor protein, presenilin-1, and presenilin-2. There are 150 genes involved with increased neuronal vulnerability to premature death in the AD brain. Among these susceptibility genes, the apolipoprotein E (ApoE) gene is the most prevalent as a risk for AD pathogenic process in which complex interactions between genetic and environmental factors are involved, leading to a cascade of pathogenic events converging in final pathways to premature neuronal death. Some of these mechanisms are common to several neurodegenerative disorders that differ depending upon the genes affected and the involvement of environmental conditions.

ApoE is a key lipoprotein in lipid and cholesterol metabolism and it is also the major risk gene for AD and many other central nervous system disorders. The pathogenic role of ApoE-4 is still to be clarified; however, diverse evidence suggests that ApoE may play pleiotropic functions in dementia and central nervous system disorders.

Key words: Alzheimer's disease, apolipoprotein E, dementia, genetic risk, neurodegeneration.

THE NUMBER OF dementia patients has increased significantly due to extended life spans. Dementia occurs in 6–15% of the elderly, causing a social problem in Japan and in other countries.[1] At present, clinicians are expected to attend 2 million dementia patients with behavioral and psychological problems of dementia, which causes a serious burden to the patients as well as to the caregivers.[2] Pharmacological treatment, mainly by anti-psychotics, works with some patients,[3–5] but is not always useful. Some opinions recommend refraining from prescribing anti-psychotics for dementia with behavioral and psychological problems of dementia,[6] and Chinese herbal medicine is recommended by some opinion leaders.[7–9] Behavioral therapy,[10] aromatherapy[11] and animal-assisted therapy[12] are sometimes useful. Different strategies are recommended by the clinical settings either at home[13] or in institutions,[14,15] targeting each type of dementia, including Alzheimer's disease (AD),[2] frontotemporal dementia,[16] diffuse Lewy body disease,[17] and vascular dementia.[18]

Considering the limited effectiveness of these interventions, researchers are now more interested in implementing early diagnosis,[19] disease-modifying therapeutics,[20,21] and even prevention of dementia.[22]

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The concept of mild cognitive impairment (MCI) and subjective cognitive impairment (SCI) have been proposed to stimulate the understanding of the earlier stage, or prodromal stage of dementia,[1] in which the interaction in the aging brain of genetic factors and lifestyle, influencing environmental factors, is the major issue to be elucidated.[23] Apolipoprotein E (ApoE) is recognized as the most powerful genetic risk factor for dementia, and it is also a key player in lipid metabolism. We believe that the clinical findings related to ApoE should be taken into consideration, in order to reconcile gene and environmental interactions in the pathogenesis of dementia, including AD. The pathogenic role of ApoE-4 is still to be clarified. The biological functions of ApoE will be reviewed with regard to dementia and other central nervous system (CNS) disorders.

Apolipoprotein E gene

The three major isoforms of human ApoE(19q13.2) (ApoE-2, ApoE-3, ApoE-4) are coded by the ε2, ε3, and ε4 alleles. Differences in the amino acid sequence at sites A (residue 112) and B (residue 158) of the ApoE molecule distinguish the ApoE-2 (Cys/Cys), ApoE-3 (Cys/Arg), and ApoE-4 (Arg/Arg) isoforms.[24,25]ApoE-3 is the most frequent isoform (wild-type), and ApoE-4 differs from ApoE-3 in a Cys-to-Arg change at position 112 (ApoE-4/Cys112Arg). ApoE-2 (Arg158Cys) is the most common isoform of the four different mutations at the E2 position with isoelectric focusing. The other three ApoE-2 isoforms are E2(Lys146Gln), E2(Arg145Cys), and E2(Arg136Ser).[26] The ApoE gene encodes a 299-amino acid polypeptide (Mw 34 200). This gene is in close proximity with the APOC1, APOC2 and GPI genes in the same region of 19q.[27] Sequence haplotype variation in 5.5 kb of genomic DNA encompassing the whole ApoE locus and adjoining flanking regions revealed the existence of 22 diallelic sites defining 31 distinct haplotypes. Sequence analysis suggested that haplotypes defining the ApoE-3 and ApoE-2 alleles were derived from the ancestral ApoE-4 and that the ApoE-3 group of haplotypes had increased in frequency, relative to ApoE-4, over the past 200 000 years. Substantial heterogeneity is present in the three classes of sequence haplotypes, with interpopulation differences in the sequence variation underlying the protein isoforms, probably explaining conflicting results when interpreting phenotypic associations with variation in the common protein isoforms.[28,29]

The ApoE alleles show a peculiar distribution throughout the world.[30] The ApoE-3 allele is the most frequent in all human societies, especially in populations with a long-established agricultural economy, such as those of the Mediterranean basin, where the allele frequency is 0.849–0.898. ApoE-4 is the ancestral allele, with a frequency that still remains higher in Pygmies (0.407), Khoi San (0.370), Papuans (0.368), Lapps (0.310), some Native Americans (0.280), Australian Aborigines (0.260), and Aborigines of Malaysia (0.240) where an economy of foraging still exists, or food supply is scarce or sporadically available. The frequency of the ApoE-2 allele fluctuates with no apparent trend (0.145–0.02) and is absent in Native Americans and very low (<1%) in southern Europeans.[30–32]

Biological function of ApoE

The best known effect of ApoE is the regulation of lipid metabolism; however, in addition to its role in the transport of cholesterol and the metabolism of lipoprotein particles,[33] ApoE may be involved in many other physiological and pathological processes, including immunoregulation, nerve regeneration, activation of lipolytic enzymes (hepatic lipase, lipoprotein lipase, lecithin : cholesterol acyltransferase), ligand for several cell receptors, neuronal homeostasis, and tissue repair.[29]

ApoE is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. The interaction of ApoE and the low-density lipoprotein (LDL) receptor controls the removal of ApoE-rich lipoproteins (very low-density lipoprotein [VLDL], chylomicron remnants, intermediate density proteins) and determines the homeostasis of cholesterol and triglycerides.[31] Some studies indicate that ApoE polymorphism variation may explain 14–17% of the genetic variability of plasma cholesterol concentrations.[31,34,35]

The three ApoE isoforms have different affinities for the LDL receptor, ApoE-3 and ApoE-4, showing similar affinities and ApoE-2 exhibiting a defective binding activity. ApoE plays a critical role in lipoprotein metabolism and plasma lipid homeostasis through its high-affinity binding to the LDL-receptor family. In solution, ApoE is an oligomeric protein, and the C-terminal domain causes ApoE's aggregation. The aggregation property presents a major difficulty for structural determination of this protein. Using protein engineering techniques, Fan *et al.* identified a monomeric, biologically active ApoE

C-terminal domain mutant. This mutant replaces five bulky hydrophobic residues in the region of residues 253–289 with either smaller hydrophobic or polar/charged residues (F257A, W264R, V269A, L279Q, V287E). These residues are critical for aggregation but may not be important for maintaining the structure, stability, and lipid-binding activity of this ApoE domain, suggesting that ApoE may use different epitopes for its aggregation property, helical structure/stability, and lipid-binding activity.[36]

ApoE-2-containing remnants and VLDL particles are slowly removed from the plasma and induce an upregulation of the liver LDL receptor and subsequent low concentration of plasma cholesterol. VLDL-ApoE-4 particles are removed from plasma faster than VLDL-ApoE-3 particles, inducing a down-regulation of the LDL receptor, and thus the VLDL-ApoE-4 phenotype is associated with higher concentration of circulating cholesterol.[31]

The ApoE genotype is an important determinant of plasma and CSF ApoE and lipid levels. The ApoE-2 allele is associated with high concentrations of ApoE and the ApoE-4 allele with lower ApoE levels.[31] Some authors have found association between ApoE-2 and ApoE-4 and high levels of plasma triglycerides as well as an association of ApoE-3 and low levels of triglycerides in the general population.[35]

It has also been suggested that individuals carrying the ApoE-4 genotype had a significantly greater increase in triglycerides accompanied by an increase in bodyweight, suggesting that obese individuals with an ApoE-4 allele might be at increased risk for developing hypertriglyceridemia and atherosclerosis; however, recent studies in the AD population clearly indicate that: (i) cholesterol levels are markedly increased in ApoE-4/4 carriers; (ii) triglyceride levels are the lowest in ApoE-4/4; and (iii) ApoE-4/4 carriers show a high atherogenic activity. These differences probably reflect the influence of endogenous factors interacting with ApoE to induce an alteration in lipid metabolism in patients with AD.[37–39]

The ApoE-4/4 and ApoE-3/4 genotypes have also been associated with high systolic blood pressure levels.[40] It has been suggested that the ApoE-2 allele may exert a protective effect on coronary atherosclerosis,[41,42] and that the ApoE-4 allele increases the risk of myocardial infarction and atherosclerosis.[31,42] After studying the association of ApoE with birthweight, Garces *et al.* suggested that the interaction of the ApoE genotype and birthweight may be an important determinant of atherosclerosis.[43]

Sullivan *et al.* studied the pattern of ApoE expression in the CNS. Immunocytochemistry on brain sections from three human ApoE targeted replacement mouse lines, wild-type mice, African green monkeys, and humans, and showed a predominantly glial pattern of ApoE expression. The levels of human ApoE protein in the hippocampus and frontal cortex were similar between targeted replacement mice and non-demented human tissue. Within a given brain region, the levels of ApoE were very similar amongst all three isoforms, which contrasts sharply with plasma, where ApoE2 levels are 16-fold higher than ApoE3 and ApoE4 levels. Across brain regions, cerebellar ApoE levels were significantly higher than cerebral ApoE levels.[44] In the human brain, ApoE-4 dose correlates inversely with dendritic spine density in the hippocampus.[45] ApoE is expressed at high levels in hepatocytes, macrophages, fibroblasts and astrocytes. Neurons also express ApoE at lower levels than astrocytes in response to various physiological and pathological conditions, including excitotoxic stress. Neuronal expression of ApoE is regulated by a diffusible factor or factors released from astrocytes, and this regulation depends on the activity of the extracellular signal-regulated kinase (Erk) pathways in neurons.[46] For many years, alterations in ApoE and defects in the ApoE gene have been associated with dysfunctions in lipid metabolism, cardiovascular disease, and atherosclerosis. An enormous number of studies, however, clearly documented the role of ApoE-4 as a risk factor for AD.

ApoE in Alzheimer's disease (AD)

In 1993 Allen Roses and co-workers found a clear association between ApoE genotypes and AD, demonstrating that the frequency of the ApoE-4 allele was significantly higher in late-onset Alzheimer's disease (LOAD).[47] Since then, many studies have confirmed this, reporting an increased frequency of the ApoE-4 allele in AD and the association of the ApoE-4 allele with LOAD and sporadic forms of AD. A protective effect of ApoE-2 for LOAD has also been proposed[48] and confirmed.[49] There is also a significant lowering of age at onset for subjects with ApoE-4/4 as compared to other ApoE genotypes.[50] ApoE-4 promotes arteriosclerosis and is less frequent in centenarians than in controls, and ApoE-2, which was associated with type III and type IV hyperlipemia, is more frequent in people with higher longevity rates.[51] The risk for AD increases from 20% to 90% and mean age at

onset decreases from 84 to 68 years with an increasing number of ApoE-4 alleles, confirming the dosage effect of the ApoE-4 allele, which in ApoE-4/4 homozygotes anticipates the age at onset to their 60s. The combination of low head circumference and ApoE-4 is also a strong predictor of early-onset AD.[52]

ApoE is found in amyloid plaques and neurofibrillary tangles (NFT) in AD brains. The accumulation of potentially pathogenic C-terminally truncated fragments of ApoE depends on both the isoform and the cellular source of ApoE. Neuron-specific proteolytic cleavage of ApoE-4 is associated with increased phosphorylation of tau and may play a key role in the development of AD-related neuronal deficits.[53] Hippocampal ApoE levels correlate with NFT formation, especially in ApoE-3/3 autopsy samples, but not in ApoE-4 carriers.[54] Monocyte-derived macrophages exhibit a significantly greater increase in nitric oxide production during immune activation in AD patients with the ApoE-4 allele. Enhanced macrophage responsiveness and increased production of nitric oxide in ApoE-4 may predispose the CNS to an increased potential for nitration and nitrosation, consistent with the reduction-oxidation imbalance and neuroinflammatory state observed in AD.[55]

ApoE may affect NFT and β-amyloid peptide (BAP) deposition in AD.[56] ApoE-4-related proteins may interfere with binding of tau to microtubules, altering tau glycation and phosphorylation.[57] The presence of ApoE-4 increases the odds ratio for cerebral amyloid angiopathy; and ApoE-4 is strongly associated with increased BAP deposition in AD.[58–60] The oxidized form of purified ApoE-4 shows a higher affinity binding to synthetic BAP and MAP2 than the ApoE-3 isoform, and probably ApoE may affect microtubule function and BAP accumulation in AD.[56,61] Carriers of ApoE-2 and ApoE-4 alleles are also more prone to recurrent cerebral amyloid angiopathy than ApoE-3/3 carriers.[62] AD ApoE-4 carriers show reduced glucose metabolism in selected brain regions.[63] There is also an ApoE-related cognitive decline in AD patients, which is more accelerated in subjects with the ApoE-4/4 genotypes. ApoE-related differences in serum ApoE levels,[64,65] blood pressure values[66] and lymphocyte apoptosis[67,68] have been demonstrated in AD. ApoE-4/4 patients are also the worst responders to different treatments.[26] ApoE-4 carriers also show a poorer brain metabolism.[63,69,70]

The ApoE-4 genotype is accompanied by lower metabolic activity in the nucleus basalis of Meynert neurons in AD and controls.[71] Dubelaar *et al.* used the size of the Golgi apparatus as an indicator of metabolic activity to show that control subjects harboring the ApoE-4 allele have reduced neuronal metabolism and show more neurons with smaller Golgi apparatus size compared with ApoE-4 non-carriers. As the disease progresses into later stages of AD (Braak V-VI stages) neuronal metabolism strongly diminishes, resulting in neurons with extremely small Golgi apparatus size, irrespective of ApoE genotype.[71]

ApoE-4 may influence AD pathology interacting with APP metabolism and BAP accumulation, enhancing hyperphosphorylation of tau protein and NFT formation, reducing choline acetyltransferase activity, increasing oxidative processes, modifying inflammation-related neuroimmunotrophic activity and glial activation, altering lipid metabolism, lipid transport and membrane biosynthesis in sprouting and synaptic remodeling, and inducing neuronal apoptosis.

A critical review in the literature provides convincing support to the hypothesis of ApoE as a major player in AD pathogenesis and risk of dementia. The major facts demonstrating that ApoE is associated with AD can be summarized as follows: (i) increased frequency of the ApoE-4 allele in AD and protective effect of ApoE-2; (ii) association of ApoE-4 with an anticipation of the age-at-onset; (iii) negative influence of ApoE on cognitive performance; (iv) deleterious associations of ApoE-4 with other genes as potential risk factors for AD; (v) ApoE and sex differences in AD; (vi) association of ApoE with BAP and tau in AD pathology; (vii) ApoE and alterations in lipid metabolism; (viii) ApoE and neuroendocrine function in AD; (ix) ApoE and behavior; (x) ApoE and brain atrophy; (xi) ApoE and survival; and (xii) ApoE in other CNS disorders.[29,72]

Association of ApoE with BAP and tau in AD pathology

The ApoE-4 isoform binds to BAP more rapidly than the ApoE-3 isoform;[73] and the ApoE-4 allele is strongly associated with increased senile plaques but not NFT in AD and in the AD Lewy body variant.[58] However, isoform-specific differences have been identified in the binding of ApoE to microtubule-associated protein tau, which forms NFT, and to BAP, a major component of senile plaques.[56] Other studies have reported that the presence of ApoE-4 is significantly associated with both BAP and NFT in autopsy

brains,[74] but the effect is differentially modified by age and gender. For instance, the effect of ApoE-4 on NFT is noted at ages 80 and above, but not between ages 60 and 79, in both genders, whereas the association between the ApoE-4 allele and senile plaques for women is found only from ages 60 to 79, but not above 80 years, with no age difference in men.[75] The amount of deposited BAP40 is significantly increased in AD brain samples with ApoE-4 allele and also in cases with the -491 AA genotype independent of ApoE-4 status, suggesting that the association between increased BAP load and alleles of the ApoE promoter polymorphism is independent of ApoE genotype.[76]

In animal models, overexpression of human ApoE-4 in transgenic mice led to an increase in plaque formation, with the association of the ApoE-4 isoform with APP and BAP in the plaques, a decrease in presynaptic terminals, and an increase in tau phosphorylation and in surrounding gliosis, all these events corresponding with major neuropathological hallmarks of AD. ApoE reduces BAP levels by 20–80% in cell cultures. ApoE may function independently of BAP, and conformational changes in its molecular structure might contribute to neurodegeneration. Characterization of the 3-D structure of ApoE shows four helix bundles in between the amino and carboxy terminal of the molecule. ApoE-4 is the most unstable isoform in terms of protein folding; ApoE-2 folds in the most stable conformation; and ApoE-3 shows an intermediate stability. The molten globule conformation linked to greater stability is acquired most often by ApoE-4 than ApoE-3 or ApoE-2, and ApoE-4 exhibits the highest tendency among these three proteins to form molten globules whose conformational features may lead to increased degradation, alterations in cell signaling, increased binding to lipids, modifications in protein–protein interactions, increased membrane binding, changes in transport through membranes, and modified interactivity with cellular receptors.[77] The structural changes in ApoE-4 seem to be related to an interaction between Arg112 and Arg61 with Glu225 that does not occur in ApoE-3 owing to the presence of a Cys residue at position 112.[74] Wild-type ApoE-4 seems to be associated with higher BAP production, more extensive disruption of the cytoskeleton, and increased lysosomal cleavage.[78–80] Astrocytes appear to play a critical role in the clearance of BAP in the brain following migration to areas of the brain rich in neurotoxic deposits. A receptor-specific uptake seems to mediate internalization and degradation, but defects in these steps associated with ApoE may impair clearance, thus favoring further accumulation of BAP and the appearance of neurodegenerative events. Expression of ApoE-3 in a transgenic model decreased the BAP load in a dose-dependent manner in PDAPP mice at 12–15 months of age, and expression of ApoE-4 led to increased deposition of BAP in these PDAPP/ApoE-knockout mice. ApoE-2 induced a marked decrease in BAP accumulation.[78] So, it appears that ApoE polymorphic variants affect the amount of BAP deposited in the brain, and ApoE is able to reduce γ-secretase cleavage of APP, lowering BAP levels. In neuronal and non-neuronal cell lines, ApoE treatment reduced BAP40 by 60–80% and BAP42 to a lesser extent (20–30%) in the conditioned media. ApoE treatment resulted in an accumulation of APP-C-terminal fragments in cell extracts and a marked reduction of APP intracellular domain-mediated signaling, consistent with diminished γ-secretase processing of APP. All three isoforms of ApoE had similar effects on BAP and APP-C-terminal fragments, and the effects were independent of the LDL receptor family.

There has been increasing interest in a potential role for fatty acids in adversely affecting organismal substrate utilization and contributing to the cardiovascular complications in insulin resistance. Fatty acids have already been implicated in regulating the expression of a number of genes in resident cells of the vessel wall. In this regard, it has been demonstrated that oleic acid increases ApoE secretion from macrophages at a locus involving post-translational glycosylation.[81]

ApoE in other forms of dementia and CNS Disorders

The distribution of ApoE genotypes clearly differs among different CNS disorders, with an accumulation of the ApoE-4 allele in dementia, especially in AD and mixed-type dementia (MXD). In early-onset AD (EOAD), the ApoE-3/4 and ApoE-4/4 genotypes account for 54.35% of the cases, and the presence of the ApoE-4 allele is more frequent in women (52.42%) than in men (35.47%). In late-onset AD (LOAD), the ApoE-4 allele is present in 55.88% of the cases (55.87% in women and 55.92% in men). According to these results, the frequency of the ApoE-4 allele is similar in women with EOAD and LOAD, but significantly higher in men with LOAD as

compared with EOAD men; however, women and men show an identical distribution in LOAD. Integrating both types of age-related AD phenotypes (EOAD+LOAD), the presence of the ApoE-4 alleles accounts for 51.38% of AD cases (54.38% in women and 45.43% in men).[29]

In pure vascular dementia (VD), secondary to severe cardiovascular and cerebrovascular disorders (e.g. stroke, atrial fibrillation, hypertension), the ApoE-4 allele is present in 37.60% of the cases, with a relative distribution similar in women (39.08%) and men (35.57%), but significantly different from the distribution pattern seen in AD. The highest accumulation of ApoE-4 carriers is observed in MXD (53.01%), with a distribution in women (58.76%) and men (45.10%) similar to that detected in AD; however, the ApoE-4/4 genotype is over-represented in both women (12.55%) and men (13.41%) with MXD. In this regard, patients with MXD exhibit the highest frequency of the ApoE-4/4 as compared with any other cluster or pathological group in the Spanish population.[29,37,39]

In AD patients with history of cerebrovascular disorders (excluding stroke), such as chronic cerebrovascular insufficiency, migraine, hypotension or dizziness, a high frequency of ApoE-4 was also found (44.71%), with identical distribution of the ApoE-4/4 genotype in women and men. Nevertheless, in patients with cerebrovascular disorders without cognitive impairment, the frequency of the ApoE-4 allele (24.65%) was similar to that of controls (24.76%), suggesting that the risk of developing dementia in patients with chronic cerebrovascular disorders may be associated with the presence of the ApoE-4 allele. In patients with different CNS disorders, including Parkinson's disease, schizophrenia, depression, anxiety and epilepsy, an increased frequency of the ApoE-4 allele was detected (41.13%), with a similar distribution in women and men, probably indicating that the ApoE-4 allele might represent a factor of brain vulnerability in different medical conditions. Finally, we found a low frequency of ApoE-4 (24.99%) in patients with stroke, practically the same as in controls and in patients with cerebrovascular disorders without cognitive deterioration. Surprisingly, a high frequency of ApoE-4 was also observed in patients with anxiety (39%), diabetes (40%) and hypertension (36%). The highest frequencies of the ApoE-4/4 genotype in decreasing order were identified in MXD, diabetes, VD, headache, and AD. The fact that patients with stroke and/or cerebrovascular disorders without cognitive impairment show a frequency of ApoE-4 similar to controls (20–30%) together with the evidence that patients with MXD and AD represent the population with the highest frequency of ApoE-4 (50–60%) suggests that the inheritance of ApoE-4 is an important risk factor in dementia in general, and that the presence of ApoE-4 in patients with cerebrovascular disorders and/or stroke may be determinant for these patients to develop dementia as a secondary event following cerebrovascular damage.[29,37,39]

Vascular dementia and cerebrovascular disorders

The frequency of the ApoE-4 allele has been found to be increased in vascular dementia (VD).[82–84] In early reports it was suggested that the increased plasma cholesterol concentrations and resulting atherosclerosis associated with ApoE-4 might contribute to VD.[82] Wieringa *et al.*[85] found a higher frequency of ApoE-4 in multi-infarct dementia, but the increased prevalence of the ApoE-4 allele was not related to serum lipid levels, and they concluded that the hypothesis that the onset of multi-infarct dementia may be precipitated by ApoE-4's mediation of higher serum cholesterol levels was not supported. Some authors did not find a great difference in ApoE-4 allele frequency between AD and VD.[86,87]

A high frequency of the ApoE-2 allele was observed in patients with cerebral amyloid angiopathy-related hemorrhage, suggesting that patients with ApoE-2 may be protected from parenchymal AD but may be susceptible to rupture of amyloid-laden vessels.[88,89] Lin *et al.* reported that ApoE-4 plays no significant role in the development of ischemic cerebrovascular disease and VD, but that ApoE-2 has a protective effect with regard to the development of ischemic cerebrovascular disorders and VD for Taiwanese-Chinese subjects younger than 65 years.[90] Greenberg *et al.* also found association between ApoE-2 and vasculopathy in cerebral amyloid angiopathy, postulating that ApoE-2 and ApoE-4 might promote hemorrhage through separate mechanisms: ApoE-4 by enhancing amyloid deposition, and ApoE-2 by promoting rupture. ApoE-2 is also a risk factor for early recurrence of cerebral amyloid angiopathy.[91] Others have reported that possession of ApoE-4 does not by itself confer an increased risk of cerebral amyloid angiopathy but may be associated with reduced longevity even in the absence of AD or

cerebral hemorrhage.[92] The ApoE-2 allele may influence the therapeutic response in some cases. For instance, there is evidence that the efficacy of i.v. tissue plasminogen activator in patients with acute ischemic stroke may be enhanced in those carrying the ApoE-2 allele.[93]

Other dementias

A high frequency of ApoE-4 has been found in Lewy body dementia[94,95] where ApoE-4 carriers also showed a greater neuritic degeneration in hippocampal CA2-3 regions. The ApoE-2/3 genotype has been associated with significantly earlier age of onset of Huntington's disease.[95] ApoE-2/2 has been associated with frontotemporal dementia, but the rarity of this genotype recommends being cautious in the interpretation of results.[96] In Chamorros with amyotrophic lateral sclerosis/parkinsonism dementia complex, the ApoE-4 allele was not found to be associated with this form of dementia and the presence of the ApoE-3 allele did not reveal any protective effect against NFT formation in this population.[97] Itabashi *et al.* compared the distribution of ApoE genotypes in a necropsy series of AD and other dementias (Parkinson's disease with dementia, progressive supranuclear palsy, Lewy body dementia, polyglucosan-body disease, Pick's disease, dementia+hydrocephalus, Wernicke–Korsakoff syndrome)[98] and found no major differences in the distribution of the ApoE-4 allele in AD and the other dementias, suggesting that the ApoE-4 allele is not predictive of AD. An increased frequency of the ApoE-4 allele was reported in bulbar-onset motor neuron disease,[99] but this could not be replicated in another study.[100] No association has been found between ApoE-4 and the incidence or the age of onset of sporadic or autosomal dominant amyotrophic lateral sclerosis.[101]

Down syndrome

Senile plaques in Down syndrome are particularly large in ApoE-4 carriers and less abundant than in AD, suggesting that pathology in Down syndrome is due to increased amyloid production and deposition with ApoE-4 probably increasing senile plaque initiation.[102] In patients with Down syndrome, ApoE-2 was associated with increased longevity and decreased frequency of dementia.[103] No ApoE-4/4 was seen in Down syndrome cases in some studies;[103] in contrast, others could not find significant differences in the distribution of ApoE genotypes between AD and Down syndrome.[104] In general terms, it appears that the frequency of the ApoE-4 allele in Down syndrome does not differ from that of the general population and that ApoE-2 may exert a protective effect.[105]

Schizophrenia

Several studies attempted to associate ApoE-4 with schizophrenia. Harrington *et al.* reported an increased frequency of ApoE-4 in schizophrenia,[106] but subsequent studies in different populations failed to replicate this finding.[107–113] However, ApoE-4 was associated with an early onset of schizophrenia,[109,114] with a reduced outcome of positive symptoms,[115,116] and with a worse prognosis in women,[112] but these results could not be replicated by others.[110,117] Some authors found that ApoE-3 might increase the risk of schizophrenia,[118] but this finding could not be confirmed.[119] Both early-onset schizophrenia[120] and a poor response to neuroleptics were associated with ApoE-2.[121] In a recent study, no differences in ApoE allele or genotype frequencies were observed in schizophrenia, although a possible association between schizophrenia in men and the ApoE-2/3 genotype was postulated.[122] In patients with paraphrenia or late-onset schizophrenia, Howard *et al.* found comparable frequencies of the ApoE-4 allele to that found in centenarians.[123]

The ApoE genotype was related to the incidence of psychiatric symptomatology.[124] The presence of one ApoE-4 allele conferred a 2.5-fold risk and the presence of two ApoE-4 alleles conferred a 5.6-fold risk for development of delusions; however, no association was found for depressive symptoms or behavioral disturbances in some studies;[121] in contrast, others have found a small increment of psychiatric symptoms and aberrant behaviors in AD patients with ApoE-4.[125]

Some authors suggest that increased levels of ApoE in the frontal cortex of schizophrenics may be associated with the pathology of schizophrenia and that antipsychotic drugs decrease ApoE levels as part of their therapeutic action.[126]

Multiple sclerosis

The ApoE-4 allele was associated with significantly faster progression of disability and more extensive axonal damage in patients with multiple sclerosis,[127]

but some studies found that ApoE-4 and/or the -491 A/T ApoE promoter polymorphism were not associated with a more rapid course of multiple sclerosis.[128] ApoE-4 was also associated with slightly earlier disease onset, but it does not constitute a risk factor for multiple sclerosis.[129–132] Niino *et al.* found no relation between ApoE and multiple sclerosis in Japan.[133]

N-acetylaspartate (NAA) is exclusively present in mature neurons, and it appears decreased in multiple sclerosis, reflecting neuronal loss, axonal loss, and generalized neuronal dysfunction. Multiple sclerosis patients with ApoE-4(+) exhibit a higher degree of disability and a lower NAA : creatine ratio than patients with ApoE-4(−)(244). ApoE-4(+) carriers have more relapses and have a 5-fold higher rate of annual brain volume loss compared to ApoE-4(−) carriers. ApoE-4(+) carriers also show an increase in individual lesions on magnetic resonance imaging. In contrast, ApoE-2 carriers show the lowest annual volume loss of brain volume.[127] These results by Enzinger *et al.* clearly demonstrate the negative influence that ApoE-4 exerts on brain volume, contributing to increasing brain atrophy in multiple sclerosis.[127]

Head injury

It has been reported that ApoE-4 may negatively influence recovery in patients with head injury. Teasdale *et al.* found that ApoE-4(+) carriers were more likely to have an unfavorable outcome 6 months after injury than ApoE-4(−) carriers.[134] ApoE-4(+) patients also have more difficulties with memory than matched patients without ApoE-4. The performance of ApoE-4(+) carriers is poor regardless of severity of injury, whereas performance in ApoE-4(−) carriers worsens in parallel with more severe injury.[135] In patients with mild to moderate traumatic brain injury the ApoE-4 allele also affects short-term recovery.[136] The frequency of the ApoE-4 allele was also found to be increased in patients with prolonged post-traumatic unawareness who did not recover consciousness. In addition, ApoE-4 was associated with BAP deposition following head injury. CSF ApoE and BAP levels decrease after traumatic brain injury, whereas CSF S100B levels increase. There is also a correlation between injury severity and the decrease in BAP after brain injury.[137]

Parkinson's disease

The ApoE-4 allele does not function as a risk factor that influences the development of AD lesions in Parkinson's disease.[138] The ApoE-4 allele frequency in Parkinson's disease patients with dementia (0.068) and in those without dementia (0.13) does not greatly differ from controls (0.102), indicating that the biological basis of dementia in Parkinson's disease may differ from that of AD (254). In general, ApoE-4 was not associated with Parkinson's disease in the Caucasian population.[139] However, the age at onset of Parkinson's disease appears to be significantly earlier in ApoE-3/4 and ApoE-4/4 carriers than in patients with the ApoE-3/3 genotype.[140]

Prion disease

Initial studies did not find association between ApoE-4 and other amyloid-forming diseases, including Creutzfeldt–Jakob disease, familial amyloidotic polyneuropathy, and Down syndrome. Subsequent studies concluded that ApoE-4 might be a major susceptibility factor for Creutzfeldt–Jakob disease.[141]

Other diseases

Increased frequency of ApoE-4 has been found in patients with inclusion body myositis.[142] The probability of moderate to severe sleep-disordered breathing (apnea/hypopnea) was reported to be significantly higher in ApoE-4(+) carriers, independent of age, sex, body mass index, and ethnicity.[142] Patients with primary dystonia harboring the ApoE-4 genotype tend to have an earlier age at onset than ApoE-4(−) carriers.[143]

Copin *et al.* have reported that two ApoE-promoter SNP previously associated with AD also modified the primary open-angle glaucoma (POAG) genotype. ApoE(-219G) is associated with increased optic nerve damage,[144] and ApoE(-491T), interacting at a highly significant level with a SNP in the myoclin gene (MYOC) promoter (MYOC-1000G), is associated with increased intra-ocular pressure and with limited effectiveness of intra-ocular pressure-lowering treatments in patients with POAG. Some studies have speculated with an increased frequency of glaucoma in AD patients; however, the studies of Copin *et al.*[145] have been criticized by Bunce *et al.*,[146] and Ressiniotis *et al.*[147] reported that ApoE is not a risk factor for developing POAG, even in patients with normal

tension glaucoma. Other studies also indicate that the ApoE genotype does not constitute a risk factor for developing POAG.[147]

Although there is no apparent association of particular ApoE genotypes with macular degeneration,[148] the inheritance of specific ApoE alleles has been linked to the incidence of age-related macular degeneration (ARMD). The ApoE-4 allele appears to be protective, or at least, to delay the age at diagnosis of the disease, whereas the ApoE-2 allele appears to have a modifier effect by bringing forward the mean age of disease diagnosis.[149,150] ApoE is an intrinsic component of drusen, the hallmark of ARMD. Age-related alteration of lipoprotein biosynthesis and processing at the levels of the retinal pigment epithelium, where ApoE can be locally synthesized, and/or Bruch membrane might be a significant contributing factor in drusen formation and ARMD pathogenesis.[151] ApoE has also been implicated in pupil dilation, and a hypersensitive pupil dilation response to tropicamide was reported in cognitively normal individuals with the ApoE-4 allele.[152] Hypersensitivity responses of the pupil to the cholinergic agonist pilocarpine and the antagonist tropicamide have also been reported in AD,[153,154] but these findings could not always be replicated.[155]

Estrogen use was associated with less cognitive decline among 2716 women (>65 years) who did not have the ApoE-4 allele, but not among women who had at least one ApoE-4 allele,[156] probably indicating that ApoE-4(+) carriers under estrogen regimens may have a higher risk of cognitive deterioration.

The ApoE-4 allele frequency is not increased in familial non-insulin-dependent diabetes mellitus (NIDDM), despite the presence of ApoE in the pancreatic islet amyloid in NIDDM.[157] In China, Liu *et al.*[158] found that: (i) the heparan sulfate proteoglycan (HSPG) T allele is a risk factor for the development of severe diabetic nephropathy in type 2 diabetic patients; (ii) the ApoE-2 allele is a risk factor for the occurrence of type 2 diabetes mellitus in the Chinese general population; and (iii) the co-inheritance of HSPG-T/ApoE-2 confers a higher risk of type 2 diabetes mellitus progression to diabetic nephropathy in Chinese.[158] In the Japanese population, the ApoE-2 is a prognostic risk factor for both the onset and progression of diabetic nephropathy in type 2 diabetes.[159]

Herpes simplex virus type 1 (HSV1) is present in certain regions of the brain in a high proportion of elderly subjects and patients with AD. It has been reported by Itzhaki and co-workers that the combination of HSV1 in the brain, and carriage of the ApoE-4 allele, was a strong risk factor for AD.[160–163] Corder *et al.* also showed that HIV-infected subjects with the ApoE-4 allele have excess dementia and peripheral neuropathy, postulating that long-term survivors of HIV infection with ApoE-4 may be at high risk for dementia and that gene–viral interaction may speed AD pathogenesis.[164] Tursen *et al.* recently reported that the presence of ApoE-2/3, high-density lipoprotein (HDL)-cholesterol levels and the absence of the ApoE-3/3 genotype can be regarded as risk factors for superficial fungal disease, especially dermatophytosis.[165]

Cardiopulmonary bypass induces a rise in cytokine release by activated monocytes. ApoE-4 and TNFB polymorphisms (TNFB-A329G) are risk factors for atherosclerosis. The presence of TNFB*A329G and ApoE-4 is associated with significantly higher releases of IL8 and TNFA, prolonged intubation, and increased transfusion in patients undergoing coronary artery bypass grafting, relative to patients without genetic variants.[166]

The ApoE-2 allele seems to be associated with the lowest reproductive efficiency and the ApoE-3 with the highest. The different total cholesterol levels associated with ApoE genotypes could have an effect on steroidogenesis and as a consequence determine the observed differential fertility.[167]

Exercise

Physical activity improves lipid levels by altering triglyceride metabolism, and ApoE facilitates triglyceride clearance by mediating lipoprotein binding to hepatic receptors. Thompson *et al.* studied the influence of ApoE variants on lipid and physiological response to exercise training in the USA.[168] This prospective study demonstrates that the serum lipid response to exercise training differs by ApoE genotype in a pattern consistent with known metabolic differences among the variants. TG were slightly higher in ApoE-2/3, whereas LDL-cholesterol was lower. TG decreased by 11% with training for the entire cohort, and 7%, 12%, and 14% for ApoE-2/3, ApoE-3/3 and ApoE-3/4, respectively. LDL-cholesterol did not change in the cohort, but decreased slightly in ApoE-2/3 and ApoE-3/3 subjects, and increased 4% in the ApoE-3/4 group. Total cholesterol/HDL and LDL/HDL decreased with training in ApoE-2/3 and ApoE-3/3, but increased in