protective role of apolipoproteins is detectable in patients with AD [20]. However, at which point in time normal metabolic conditions switch to those disease-related remains unknown, mainly due to the need of a large number of well-characterized cases in order to achieve statistical meaning. DS brains at different ages represent the various stages seen in AD pathology, [18,34], and plasma samples reconstructed from a large cohort of DS patients can mimic the pre-symptomatic and symptomatic AD-like metabolic conditions. We hypothesized that an alteration of the lipoprotein–sAβ interaction is able to initiate and/or maintain the cascade favoring Aβ oligomerization. To verify this hypothesis and extend previous observations [20,36], the effect of aging in the lipoprotein–sAβ interaction was examined in DS patients, age-matched normal controls (NCs) and AD cases. To further assess the in vivo relevance of lipoprotein-free brain sAβ in AD pathology, we have characterized the dissociation of sAβ from its lipidic environment in AD and control brain parenchyma.

## 2. Materials and methods

### 2.1. Patients

After informed consent was given, blood samples (7 ml) were collected in 0.1% EDTA from 178 Down's syndrome (DS; ages 1–64 years), 100 Alzheimer's disease (AD; ages 46–102 years), and 241 normal controls (NC; ages 6–105 years) after 12-h fasting. None of the individuals in either group had a history of liver disease or other condition that might have affected their lipoprotein profile and none were taking drugs known to affect lipid metabolism. DS patients were characterized through clinical evaluation; trisomy 21 was confirmed by chromosomal analysis. The degree of cognitive impairment was assessed with the Mini Mental State Examination (MMSE) [4]. Accordingly, AD patients were subgrouped into those with mild AD (MMSE, >20, $n$ = 27), moderate AD (MMSE, 11–20, $n$ = 29), or severe AD (MMSE, <11, $n$ = 44).

### 2.2. Lipoprotein separation and depletion

After separation of plasma from blood cells, lipoprotein-depletion was carried out by preparative sequential density flotation ultracentrifugation using 600 μl of plasma and a protocol previously described [20] in 101 out of 178 patients with DS, 100 out of 100 patients with AD, and 103 out of 241 NCs. Briefly, the density of the collected plasma were adjusted to 1.25 g/ml using KBr and ultracentrifuged at 100,000 rpm for 8 h at 16 °C using a Hitachi RP100AT rotor. The infranatant at the density of 1.25 g/ml, named lipoprotein-depleted plasma (LPDP), as well as the floated lipoproteins were subjected to ultrafiltration using a 3 kDa cut-off membrane (Microcon 3; Amicon, Inc.) and stored until use either frozen or at 4 °C.

### 2.3. Tissue extraction

The AD brains used in this study were selected from patients that fulfilled the CERAD criteria [22] and classified on the basis of classic neuropathology (presence of senile plaques and neurofibrillary tangles). Gray matter was dissected free of vessels. Cerebral cortex (0.25 mg) from six AD and four control brains was homogenized with a motor-driven Teflon/glass homogenizer (20 strokes) in 1 ml of Tris-buffered saline (TBS, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl) and ultra-centrifuged using a Hitachi RP100AT rotor at 100,000 × $g$ for 1 h. The resultant supernatant (named TBS soluble fraction) was subjected to size-exclusion chromatography and immunoblot, and sAβ species were quantitated via specific ELISAs. The pellet, after being washed once, was further extracted with 1 ml of 70% formic acid (FA) and the homogenate ultra-centrifuged as described above. The resultant supernatant (named FA soluble fraction) was also subjected to size-exclusion chromatography, immunoblot analysis, and ELISAs.

### 2.4. Size-exclusion chromatography

The Aβ species either in TBS or 70% FA, obtained as described above, were fractionated on a Superose 12 size-exclusion column (1 cm × 30 cm, Pharmacia Biotech., Uppsala, Sweden) equilibrated with the corresponding mobile phase solution at a flow rate of 0.5 ml/min. Twenty-eight fractions of 1 ml each were collected and analyzed. To determine where Aβ eluted, 100 μl aliquots either from saline-soluble fractions or FA extracts (diluted 1000-fold with 1 M Tris–HCl, pH 8.0) were analyzed by ELISA. For evaluation of lipids, total cholesterol was enzymatically measured using a standard kit (Wako). Under our experimental conditions, plasma lipoproteins were eluted in fractions 7–14 while fractions 15–28 contained cholesterol-free proteins.

### 2.5. Immunoblot analysis

In order to characterize the distribution of monomeric and oligomeric Aβ eluted on the size-exclusion chromatography, aliquots of 100 μl FA-soluble supernatants or 500 μl saline-soluble supernatants desalted using a 3 kDa cut-off membrane were dried in a rotary vacuum and separated on 10% Tris/Tricine SDS–PAGE using standard protocols. The resulting Aβ species were transblotted onto immobilon P (Millipore) for 45 min at 400 mA using 10 mM CAPS, pH 11, containing 10% methanol [11]. The membranes were blocked with 5% low-fat milk in PBS containing 0.05% Tween 20 and incubated with monoclonal 6E10 1:1000 (anti-Aβ1–16), followed by horseradish peroxidase-labeled sheep anti-mouse F(ab')$_2$ 1:2000 (Amersham). Immunoblots were visualized with an enhanced chemiluminescence (ECL) detection kit and exposed to Hyperfilm ECL (Amersham).

### 2.6. Aβ40 and Aβ42 quantitation

Sandwich ELISA [1,32] was used to specifically quantitate whole plasma or LPDP Aβ species, as previously described in detail [20]. Microplates were pre-coated with monoclonal BNT77 (IgA, anti-Aβ11–28, specific for Aβ11–16) and sequentially incubated with 100 μl of samples followed by horseradish-peroxidase-conjugated BA27 (anti-Aβ1–40, specific for Aβ40) or BC05 (anti-Aβ35–43, specific for Aβ42 and Aβ43) [1,32]. For the analysis of brain Aβ species, 100 μl of saline-soluble Aβ species were directly subjected to ELISA, whereas the insoluble Aβ samples in the form of 70% formic acid extracts, were neutralized with 1 M Tris–HCl (pH 8.0) and diluted 1:1000 prior to ELISA. The resulting values were corrected with the wet weight of the brain to be finally expressed as pmol/g. The plates were normalized to each other by inclusion of three standard plasma samples on all plates.

### 2.7. Statistical analysis

Non-parametric methods (Kruskal–Wallis test or Mann–Whitney test) were used. Statistical significance was set at $P < 0.05$. Significant differences among groups were further analyzed using Dunnet's post test for multiple comparisons and correlation studies were made with Spearman's rank correlation. When necessary, a logarithmic transformation was used to achieve a normal distribution for data obtained. All statistical evaluations were performed with the GraphPad Prism, Version 3.0 (GraphPad Software, San Diego, CA).

## 3. Results

### 3.1. Aβ levels in NC

Spearman's rank analysis of Aβ42 in the normal controls group revealed no statistic correlation with age for either whole plasma (Fig. 1a; $n = 241$) or LPDP (Fig. 1b; $n = 103$) levels of Aβ42 ($P = 0.05$ versus 0.4902) with a mean value ± S.D. of 15.6 ± 2.1 fmol/ml versus 2.0 ± 1.4 fmol/ml, respectively. In relative terms, Aβ42 in lipoprotein-free fraction represented ~3% of total plasma Aβ which remained unmodified until age 50's, followed by a slow but steady decline, 2.1–1.4% between the ages 60 and 100, respectively (Fig. 1c). In contrast, a statistical significant age-dependent increase of Aβ40 over age 70–90's was observed in both whole plasma (Fig. 1d; $n = 241$, $P = 0.0003$) and LPDP (Fig. 1e;$n = 113$; $P = 0.0009$), with a mean value ± S.D. (fmol/ml) as follows—whole plasma: 61.5±32.2 (<60, $n = 172$) versus 103.8 ± 41.5 (>60, $n = 69$); LPDP: 6.7 ± 3.7 (<60, $n = 54$) versus 10.9 ± 6.0 (>60, $n = 59$), respectively. In relative terms, lipoprotein-free Aβ40 represented ~8% of total plasma Aβ, and remained almost unchanged until the age 90 (Fig. 1f). In healthy centenarians, all values tend to slightly fall.

### 3.2. Aβ levels in AD

In order to identify potential differences between AD patients and age-matched normal controls, the analysis of whole-plasma and lipoprotein-free Aβ species as a function of the degree of cognitive impairment (MMSE scores grouped as >20, 20–11, and <11) was performed. Whole plasma sAβ42 remained slightly elevated in all of three phases of the disease compared with the age-matched normal control group (Fig. 2a), although the differences were not statistically significant ($P > 0.05$). However, as depicted in Fig. 2b, Kruskal–Wallis non-parametric analysis revealed that lipoprotein-free Aβ42 was significantly increased in the initial stages of the disease in comparison with normal control values ($P < 0.01$ for mild AD and $P < 0.001$ for moderate AD, for post hoc comparisons). Mann–Whitney test revealed that Aβ42 levels in LPDP declined with the progression of AD from mild or moderate to severe ($P = 0.018$ for mild and $P = 0.016$ for moderate). In relative terms, the percentage of lipoprotein-free sAβ42 in whole plasma sAβ was only significantly increased in mild AD when compared with age-matched normal controls (Fig. 2c; $P < 0.05$, for post hoc comparisons). The relative percentage was significantly lower in severe AD when compared with mild AD ($P = 0.0316$), but there were no significant differences with the other AD subgroups tested ($P < 0.05$). In the case of sAβ40, the values for whole plasma and lipoprotein-free Aβ40, as well as the relative percentage were similar to normal control values in all stages of the disease, with no statistical differences among the groups tested ($P > 0.05$, Fig. 2d–f).

### 3.3. Aβ levels in DS

In DS patients, aging appeared to act slightly different in the homeostasis of plasma sAβ levels (Fig. 3). The concentration of whole plasma sAβ42 (Fig. 3a) remained stable until age 40's followed by a significant decrease in the 50's ($P < 0.001$, post hoc comparisons). A similar trend was observed for the LPDP sAβ42. Kruskal–Wallis test revealed a significant decline of lipoprotein-free sAβ42 ($P < 0.001$, post hoc comparisons) in those patients age 40–60's compared with those age 30's or below (Fig. 3b). Almost identical tendency was observed in the age-related change of whole plasma and lipoprotein-free sAβ40 (Fig. 3d and e). In relative terms, the increase of sAβ42 seen in DS at early age may represent a pre-symptomatic AD phase, whereas the subsequent decline may well parallel the disease progression (Fig. 3c and f). This temporal profile obtained in individuals with DS correlate with the values obtained in AD patients with different MMSE scores, although the baseline level appeared to be slightly higher in DS subjects than in AD individuals. Kruskal–Wallis test revealed that in DS teenagers the dissociation of both sAβ42 or sAβ40 from lipoprotein particles is significantly favored (Fig. 3c and f), suggesting that the increase in lipoprotein-free sAβ species

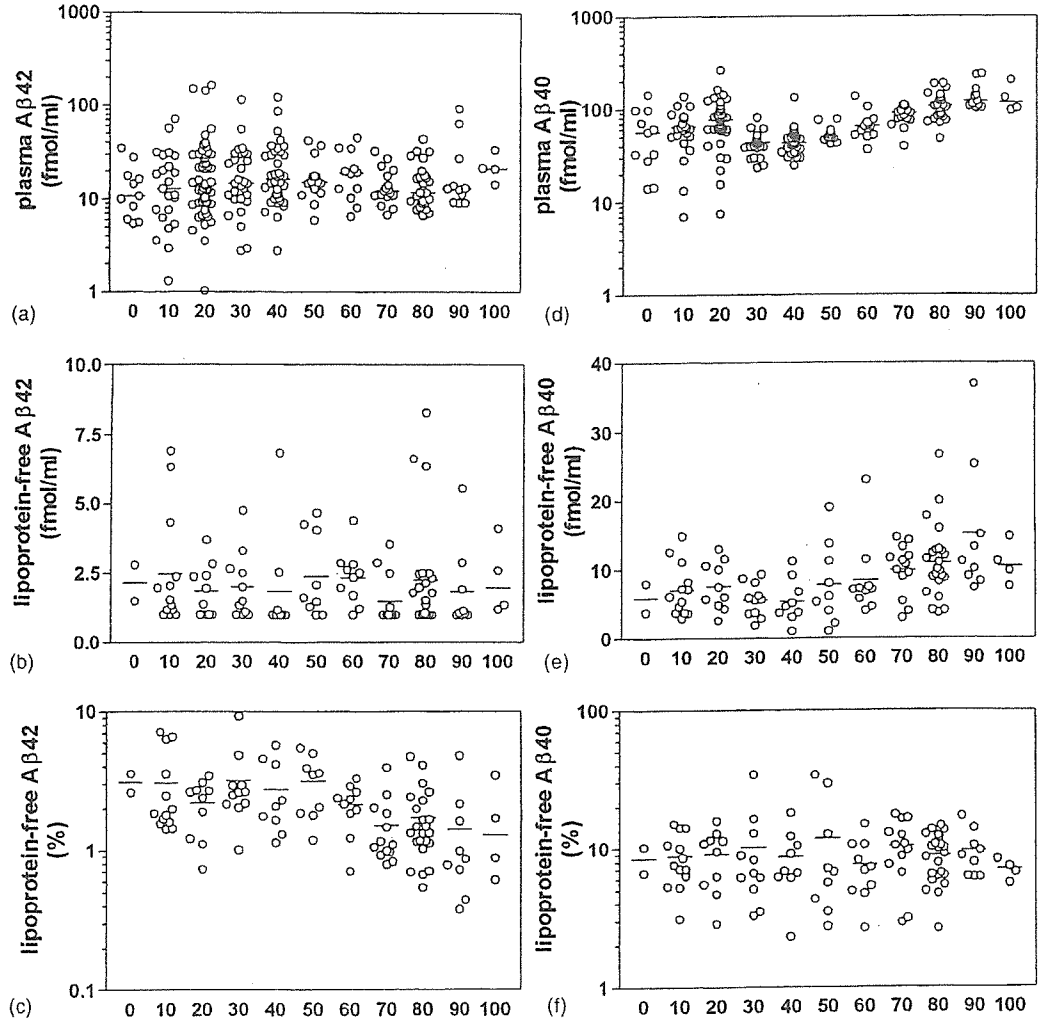


Fig. 1. Age-related changes of plasma and LPDP sAβ species in normal controls. The concentration of sAβ species was measured by captured ELISA, as described in Section 2. (a, d) Quantitation of whole plasma sAβ42 and sAβ40; (b, e) quantitation of lipoprotein-free sAβ42 and sAβ40. The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ are indicated in (c) and (f), respectively. Horizontal bars indicate the median values.

may be associated with the pre-morbidity of metabolic conditions in AD and DS.

### 3.4. *Aβ levels in brain parenchyma*

In order to further assess the potential in vivo relevance of lipoprotein-free sAβ in AD pathology, the lipoprotein-associated and lipoprotein-free sAβ species were characterized in cortical brain samples from six AD individuals and four normal controls. Samples were sequentially extracted with TBS pH 7.4 and 70% FA, and the resulting fractions analyzed via size-exclusion chromatography, immunoblot, and ELISA. In all the AD cases tested (upper half of each panel), a different gel-filtration pattern was observed for either saline-soluble or FA-extractable fractions (Fig. 4a and d, upper halves, respectively). Total cholesterol was detected in saline-soluble fractions 7 and 8 as well as in FA-extractable fraction 8. In the TBS samples, monomeric soluble Aβ immunoreactivity (Fig. 4b, upper half) was present in two fractions of different molecular mass: (i) >200 kDa, a fraction enriched in total cholesterol content exhibiting a retention time consistent with the molecular mass of lipoprotein particles (Fig. 4b, upper half, fraction 8), and (ii) 4–8 kDa, fractions containing predominantly monomeric Aβ (fractions 15 and 16) but also detectable levels of dimeric Aβ (fraction 15). ELISA analysis of the saline-soluble Aβ (Fig. 4c, upper half) identified the presence of sAβ40 and sAβ42 in either fraction. The following quantitative values were obtained—fraction 8: 95.14 fmol/ml for Aβ40 and 60.51 fmol/ml for Aβ42; fractions 15 and 16: 375.05 fmol/ml for Aβ40 and 3.07 fmol/ml for Aβ42.

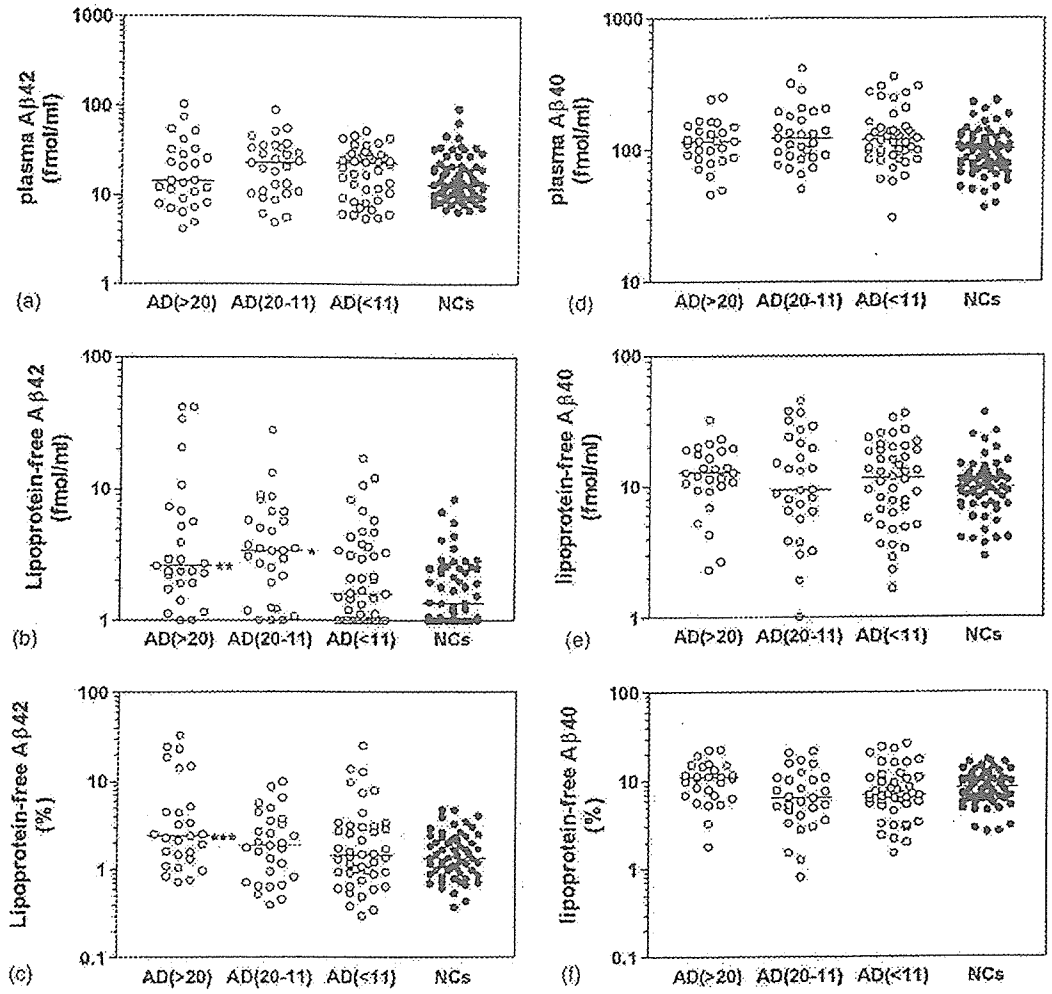


Fig. 2. Variations of plasma and LPDP sAβ species according to the severity of dementia. AD patients (○) were divided into three groups as a function of MMSE; mild AD (MMSE, >21, $n = 27$), moderate AD (MMSE, 11–20, $n = 29$), and severe AD (MMSE, <11, $n = 44$). Quantitation of whole plasma sAβ42 and sAβ40 are indicated in (a) and (d), respectively; lipoprotein-free sAβ42 and sAβ40 are depicted in (b) and (e), respectively. The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ is shown in (c) and (f), respectively. Horizontal bars indicate the median values. The statistical significance compared with age-matched control group (●, NCs; $^{*}P < 0.001$, $^{**}P < 0.01$, $^{***}P < 0.05$).

Formic acid extractable Aβ isolated from AD brain was identified in several chromatographic fractions. Fig. 4d and e depict a representative elution profile and the corresponding immunoblot analysis employing anti-Aβ monoclonal 6E10. Monomers were identified in fractions 14–15, dimers were present in fractions 12–15 and trimers in fractions 12–14 while larger molecular mass components characteristic of aggregated Aβ species were detected in fractions 11–14. No Aβ immunoreactivity was evident in cholesterol-containing fractions 7–10. Although so much immunoreactivity in fractions 11–14 and less in fraction 15, the ELISA analysis of each fraction revealed the highest Aβ levels in fraction 15, followed by fractions 14 and 16. Consistent with the previous reports showing that the BNT77-based ELISA may not detect oligomeric, SDS non-dissociable Aβ species [2,5], our ELISA failed to capture FA-extractable oligomeric Aβ species, indicating that the values obtained may represent the amount of Aβ monomers.

In all the NC cases tested (lower half of each panel), a similar gel-filtration pattern, although less amount, was observed for either saline-soluble or FA-extractable fractions when compared with AD cases (Fig. 4a and d, lower halves, respectively). Total cholesterol was detected in saline-soluble fractions 7–10 as well as in FA-extractable fraction 8. No Aβ immunoreactivity was detected in either saline-soluble or FA-extractable fractions (Fig. 4b and e, lower halves, respectively). Very little lipoprotein-associated or lipoprotein-free sAβ species was detected in four plaque-free control brains (Fig. 4c, lower half), whereas formic acid extractable Aβ was negligible in any control brains (Fig. 4f, lower half).

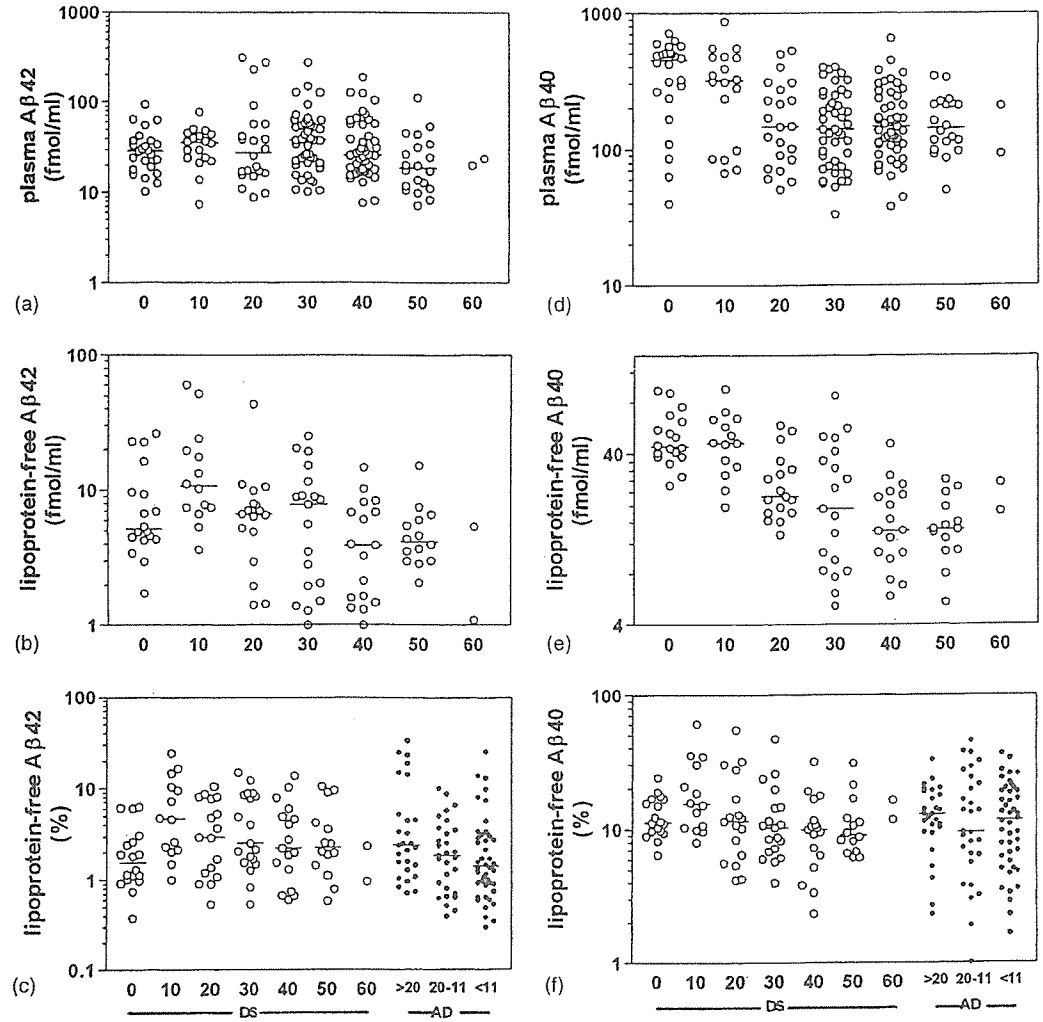


Fig. 3. Age-related changes of plasma and LPDP sAβspecies in Down's syndrome. Quantitation of whole plasma sAβ42 and sAβ40 are indicated in (a) and (d); lipoprotein-free sAβ42 and sAβ40 are shown in (b) and (e). (c, f) The percentage of lipoprotein-free sAβ42 and lipoprotein-free sAβ40 relative to whole plasma sAβ in Down's syndrome (○) and Alzheimer's disease (●). AD patients were divided into three groups according to the MMSE, as indicated in Fig. 2.

## 4. Discussion

Our data suggest that the normal lipoprotein–sAβ40 interaction in plasma is under more strict control than sAβ42 throughout the entire human life span. While Aβ40 values remain almost constant, the lipoprotein–sAβ42 interaction appeared to be reshaped in normal controls over-60's. The values obtained in sporadic AD patients remained increased in comparison with age-matched controls over-60's, indicating that sporadic AD patients, particularly mild AD, are less protected by lipoproteins. Due to the well known association of sAβ with HDL particles [14] and its low excretion into urine [8], it is conceivable that sAβ catabolic/excretory pathway(s) may follow those of lipoprotein particles. In this sense, functional decline of lipoprotein particles to reshape sAβ metabolism may provide not only the metabolic conditions to initiate and/or accelerate the cascade favoring Aβ oligomerization, but also result in reduced clearance of amyloidogenic lipoprotein-free sAβ peptides from the brain. Inadequate clearance of amyloidogenic lipoprotein-free Aβ may vary among AD and/or DS patients, resulting in the fluctuated values found in plasma studied.

It has been reported that abundant diffuse Aβ42 plaques are already present in teenage DS subjects [31]; almost every patient, aged 30 years and older, further develop Congo-red positive senile plaques [17,37]. In order to conduct further investigations, we selected to study DS patients whose brains at different ages represent pre-AD and various stages of the AD pathology. Our data suggests that in DS patients, changes in metabolic conditions favor the dissociation of sAβ from its lipidic environment during early age, resulting

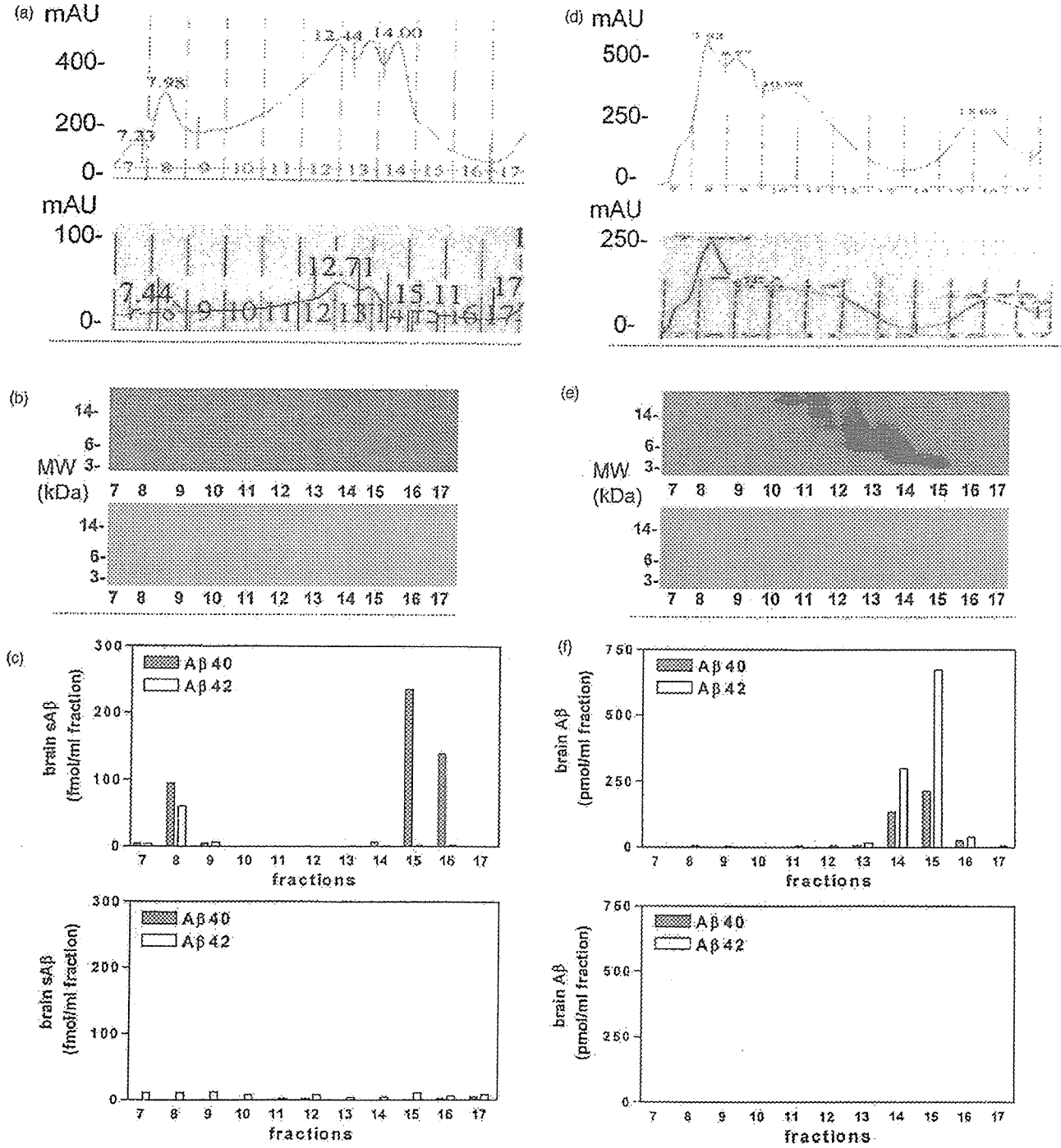


Fig. 4. Characterization of brain soluble Aβ/Aβ species in Alzheimer's disease and normal control individual. Elution profiles of saline-soluble and saline-insoluble, formic acid extractable Aβ are indicated in (a) and (d). Immunodetection of eluted Aβ species are shown in (b) and (e). (c, f) The presence of Aβ42 and Aβ40 in the different fractions. All upper half of panels are a representative result of extractions carried out in six AD cases. All lower half of panels are a representative result of extractions carried out in four NC cases.

in increased levels of amyloidogenic lipoprotein-free sAβ species, both Aβ40 and Aβ42, in plasma. Free-sAβ values decrease as the disease progresses, likely mirroring the on-going AD pathology in the DS brains. This is in agreement with our published data [12] indicating that highly significant decrease of plasma and CSF Aβ are linked to the marked deposition of Aβ in Tg2576 mice brain.

Kuo et al. isolated and quantitated brain sAβ via ultracentrifugation and molecular sieving [15] and found a continuous distribution of monomeric and oligomeric

sAβ ranging from <10 to >100 kDa. It is possible that the former represents lipoprotein-free sAβ and the latter, lipoprotein-associated sAβ species. Fagan et al. [3] reported that this dissociation also occurs in the CSF where sAβ is also associated with lipoprotein particles [13]. Our experiments using 10 brains (six AD and four NCs) correlate with those results; both sAβ species were successfully eluted from a gel-filtration matrix, suggesting that the dissociation of sAβ from lipoprotein particles occurs in brain parenchyma. It is not known whether brain specific chaperones further modulate the conversion of lipoprotein-free sAβ into amyloid fibrils via oligomeric intermediates. However, it is relevant to note that lipoprotein-free brain sAβ forms native dimers in AD brains, a feature consistently found in the six AD brains processed. The presence of less soluble free-sAβ versus much more Aβ42 amyloid in AD brains suggests that the former specie could be highly amyloidogenic in vivo, resulting in the fast conversion from the former to the latter. Alternatively, it can be speculated that our BNT77-based ELISA specific for Aβ monomer failed to detect the presence of soluble dimeric sAβ42. As discussed by several investigators, soluble Aβ oligomers appear to be the pathological amyloidogenic molecule [9–11,16,25–27,38,39]. The present data support our hypothesis that the dissociation of sAβ from or the lack of association with lipoprotein particles constitutes a potential mechanism to initiate and/or accelerate the cascade favoring Aβ oligomerization in brain. In this regard, the presence of dimeric lipoprotein-free sAβ in AD patients but not in NCs suggests that lipoproteins are not innocent bystanders but rather major determinants to balance sAβ homeostasis in biological fluids and brain parenchyma.

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# Brain-derived neurotrophic factor gene polymorphisms and Alzheimer's disease

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**Summary.** Several lines of evidence have made brain-derived neurotrophic factor (BDNF) an important candidate gene conferring risk for Alzheimer's disease (AD). Recently, three studies reported an association between two single-nucleotide polymorphisms (SNP) – i.e., C270T and G196A – in the BDNF gene and AD. This attempt to confirm these associations in a larger AD sample included examination of the linkage disequilibrium of these two SNPs. Comparison of 487 Japanese AD subjects with 471 cognitively normal elderly controls showed higher frequencies of the G allele (60.5 vs. 55.5%, $p = 0.028$) and of both the GG and GA genotypes (85.8 vs. 79.8%, $p = 0.025$) of the G196A polymorphism in AD subjects than in controls and higher frequency of the T allele of the C270T polymorphism in AD subjects who were negative for apolipotrotein E4 (2.0 vs. 4.4%, $p = 0.035$) or positive for AD family history (2.8 vs. 7.1%, $p = 0.046$). These findings suggest that BDNF gene polymorphisms play some role in the development of AD.

**Keywords:** Alzheimer disease, apolipoprotein E (ApoE4), brain-derived neurotrophic factor, case-control study, genetic association, single nucleotide polymorphism (SNP).

## Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by loss and atrophy of basal forebrain cholinergic neurons and the limbic structures (Terry, 1994). The genetics of AD is complex, and mutations of the genes encoding presenilin-1 (Sherrington et al., 1995), presenilin-2

(Levy-Lahad et al., 1995), and amyloid precursor protein cause the relatively rare, early-onset, autosomal dominant familial form of AD (Goate et al., 1991). The ε4 allele of the apolipoprotein (ApoE) gene (ApoE4) is the major known genetic risk factor for late-onset, sporadic AD (Saunders et al., 1993). However, because these genetic markers cannot explain the overall genetic susceptibility, it is clear that additional genes are involved in the development of AD.

Brain-derived neurotrophic factor (BDNF) protects cholinergic neurons of the basal forebrain and neurons in the hippocampus from ischemia-induced neuronal cell death (Pringle et al., 1996). Reduced mRNA expression of the BDNF protein has been observed post mortem in the parietal cortex of patients with AD (Holsinger et al., 2000), and lower levels of BDNF protein have been reported in the entorhinal cortex (Narisawa-Saito et al., 1996) and in the hippocampus and parietal cortex (Hock et al., 2000). Although another study reported increased BDNF level in the AD brain (Durany et al., 2000), the conflict in these lines of evidence suggest the need to investigate the BDNF gene as an important candidate for AD development.

To our knowledge, only three studies have examined the association between BDNF gene polymorphisms and AD (Kunugi et al., 2001; Riemenschneider et al., 2002; Ventriglia et al., 2002). Two of them found a significant association between the T allele of the C270T polymorphism in the non-coding region of BDNF gene and AD (Kunugi et al., 2001; Riemenschneider et al., 2002). The third, examining an association between G196A (val66met) polymorphism (dbSNP number rs6265) and AD, showed overrepresentation of the GG genotype in AD, independent of ApoE4 status (Ventriglia et al., 2002). The G196A polymorphism is located in the 5′ BDNF precursor peptide (proBDNF) sequence that is proteolytically cleaved to form the mature protein posttranslationally (Seidah et al., 1996).

This study was designed to examine the associations between the C270T and G196A BDNF gene polymorphisms and AD in a larger sample than had previously been studied, as well as to examine the linkage disequilibrium between the two polymorphisms. To eliminate the possibility of racial differences and address the highly heterogeneous etiology of AD, we limited this case-control study to Japanese subjects.

## Material and methods

The Ethics Committee of the National Institute on Alcoholism, Kurihama National Hospital (now National Hospital Organization, Kurihama Alcoholism Center) approved this study, and all participants or their families gave informed consent.

### *Subjects*

The AD group consisted of 487 Japanese patients (147 males and 340 females; mean age 76.1 ± 8.9 years) who met National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for "probable" AD (McKhann et al., 1984). The age at onset of obvious cognitive dysfunction, including memory problems, was obtained from spouses or relatives and served to identify the age at onset of AD. Among the AD cases, 101 (32 males and 69 females)

were identified as having early onset (age <65 years) of the disease, and 386 (115 males and 271 females) were identified as late-onset cases (age ≥65 years). The subjects' family background information was also obtained from their spouses or relatives, and the family histories of AD patients who had first-degree relatives with AD were considered positive. The cases identified as familial AD numbered 35, while 443 were identified as sporadic; the family history could not be ascertained for the remaining 9 AD subjects because of the uncertainty of the information.

The control group consisted of 471 elderly, unrelated Japanese (150 males and 321 females; mean age 75.2 ± 6.1 years). To evaluate their cognitive function, we administered the Mini-Mental State Examination (MMSE) (Folstein et al., 1975) and excluded subjects whose MMSE scores were less than 25.

### *Genotyping*

DNA was extracted from peripheral blood according to standard procedures. A pair of primers (forward, 5′-ATC CGA GGA CAA GGT GGC-3′; and reverse, 5′-CCT CAT GGA CAT GTT TGC AG-3′) was generated. Polymerase chain reaction (PCR) amplification was performed using 2.0 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 10 pmol of each primer according to the manufacturer's instructions (final volume, 20 μl). After activation of AmpliTaq Gold DNA polymerase for 9 min at 95°C, we subjected the reaction mixture to 35 cycles (94°C, 60°C, and 72°C for 30 s each), followed by a final extension at 72°C for 10 min. The 300-bp amplification product was purified by ethanol precipitation and digested with 10 units of *Pml I* at 37°C overnight. The resulting DNA fragments were separated on a 2% agarose gel and visualized with ethidium bromide staining. The A allele of BDNF G196A polymorphism was not digested by *Pml I*, but the G allele showed two bands, at 180 bp and 120 bp.

The BDNF C270T polymorphism was genotyped by a slight modification of a procedure described elsewhere (Kunugi et al., 2001). Briefly, we generated a pair of primers (forward, 5′-CAG AGG AGC CAG CCC GGT GCG-3′; reverse, 5′-CTC CTG CAC CAA GCC CCA TTG-3′). We performed PCR using HotStarTaq DNA polymerase (QIAGEN, Valencia, CA) and 10 pmol of each primer in a final volume of 50 μl. After activation of HotStarTaq DNA polymerase for 15 min at 95°C, we subjected the reaction mixture to 35 cycles of PCR (94°C for 60 s, 64°C for 60 s, and 72°C for 30 s each), followed by a final extension at 72°C for 10 min. The 233-bp amplification product was purified by ethanol precipitation and digested with 10 units of *Hinf I* at 37°C overnight. The resulting DNA fragments were separated on a 13.3% polyacrylamide gel and visualized with ethidium bromide staining.

ApoE genotypes were determined by a PCR-RFLP procedure described by Wenham et al. (1991).

### *Statistics*

Statistical calculations were done using a SAS package (Statistical Analysis System, SAS Institute, Cary, NC) to detect significant differences with the chi-square test and Student's *t*-test. Odds ratios (OR) with 95% confidence interval (CI) were calculated to compare the groups' allele frequencies. We used the Power and Precision program (Borenstein et al., 1997) to calculate the study's statistical power. To assess whether linkage disequilibrium exists between the two polymorphisms of the BDNF gene, we performed statistical tests using a previously described method (Matsushita et al., 2001) and the actual calculations and its statistical significance were made using the ASSOCIAT program downloaded form the website of Dr. J. Ott (http://linkage.rockefeller.edu/software/linkage).

## Results

Because of the proximity of the G196A and C270T polymorphisms in the BDNF gene, our effort to determine linkage disequilibrium between the two

Table 1. Linkage disequilibrium between the C270T and G196A polymorphisms of the BDNF gene in control subjects

| G196A genotypes | C270T genotypes | | |
|---|---|---|---|
| | CC | CT | TT |
| AA | 98 | 0 | 0 |
| AG | 209 | 14 | 0 |
| GG | 131 | 19 | 0 |

loci showed incomplete disequilibrium in control subjects ($D = 0.0156$, $\chi^2 = 20.474$, $df = 4$, $p < 0.001$) (Table 1). Therefore, we compared the distribution of each polymorphism separately.

The genotypes of the G196A polymorphisms were in Hardy-Weinberg equilibrium in controls as well as in AD subjects (Table 2). The frequencies of the GG and GA genotypes and the G allele of the G196A polymorphism were significantly higher in AD subjects than in controls (Table 2). When we set the true difference in GG genotype frequency between AD and controls in this comparison at 0.16 (0.65 − 0.49), to coincide with that in a previous report (Ventriglia et al., 2002), we achieved a statistical power of 100%. The odds ratio of the G allele for AD was 1.23 (95% CI, 1.02–1.47). Because the GG and GA genotypes were overrepresented in AD subjects, we combined these genotypes and compared the combined frequencies in AD patients and controls. The risk for AD in the presence of both GG and GA genotypes (OR) was 1.59 (95% CI, 1.13–2.23), differing significantly ($\chi^2 = 7.33$, $P = 0.0068$) from that of the risk for AD posed by the G allele.

When we divided our AD subjects according to their ApoE4 status, the presence or absence of family history of AD, and age at onset and then compared the genotype and allele frequencies of the G196A polymorphism, we found no significant differences in the distribution of G196A genotypes and alleles in AD patients with and without the ApoE4 carrier, with and without an AD-positive family history, or with early-onset versus late-onset AD.

The genotypes of both C270T polymorphisms were in Hardy-Weinberg equilibrium in controls as well as in AD subjects (Table 3). Because we found the TT genotype in only one sporadic AD subject, we combined the CT and TT genotypes for further analysis. There were no significant differences in C270T polymorphism distribution between overall AD and control subjects. Setting the true difference in T-allele frequency between the AD cases and controls at 0.04 (0.06 − 0.02), as in a previously reported study (Kunugi et al., 2001), yielded a statistical power of 99%.

Subsequent comparison of the AD subjects' C270T genotype and allele distributions by ApoE4 status, family history of dementia, and age at AD onset showed that the AD subjects who lacked ApoE4 and those who had a family history of AD had significantly higher frequencies of the T allele and of the CT (and TT) genotypes.

**Table 2.** Genotype and allele frequencies of the BDNF gene G196A polymorphism in Alzheimer's disease cases and controls

| | Alzheimer's disease cases | | | | | | | Controls (n = 471) N (%) |
|---|---|---|---|---|---|---|---|---|
| | ApoE4 (+) (n = 248) N (%) | ApoE4 (−) (n = 239) N (%) | Family history (+) (n = 35) N (%) | Family history (−) (n = 443) N (%) | Early onset (<65 yr) (n = 101) N (%) | Late onset (≥65 yr) (n = 386) N (%) | Total (n = 487) N (%) | |
| Genotype | | | | | | | | |
| GG | 88 (35.5) | 83 (34.7) | 10 (28.6) | 159 (35.9) | 34 (33.7) | 137 (35.5) | 171 (35.1) | 150* (31.9) |
| GA | 126 (50.8) | 121 (50.6) | 21 (60.0) | 220 (50.0) | 52 (51.5) | 195 (50.5) | 247 (50.7) | 223 (47.4) |
| AA | 34 (13.7) | 35 (14.6) | 4 (11.4) | 64 (14.5) | 15 (14.9) | 54 (14.0) | 69 (14.2) | 98 (20.8) |
| Allele | | | | | | | | |
| G | 302 (60.9) | 287 (60.0) | 41 (58.6) | 538 (60.7) | 120 (59.4) | 469 (60.8) | 589 (60.5) | 523† (55.5) |
| A | 194 (39.1) | 191 (40.0) | 29 (41.4) | 348 (39.3) | 82 (40.6) | 303 (39.3) | 385 (39.5) | 419 (44.5) |

* Total AD vs. control: $df = 2$, $\chi^2 = 7.37$, $p = 0.0251$. † Total AD vs. control: $df = 1$, $\chi^2 = 4.82$, $p = 0.028$

**Table 3.** Genotype and allele frequencies of the BDNF gene C270T polymorphism in Alzheimer's disease cases and controls

| | Alzheimer's disease cases | | | | | | | Controls (n = 471) N (%) |
|---|---|---|---|---|---|---|---|---|
| | ApoE4 (+) (n = 248) N (%) | ApoE4 (−) (n = 239) N (%) | Family history (+) (n = 35) N (%) | Family history (−) (n = 443) N (%) | Early onset (<65 yr) (n = 101) N (%) | Late onset (≥65 yr) (n = 386) N (%) | Total (n = 487) N (%) | |
| Genotype | | | | | | | | |
| CC | 238 (96.0) | 219 (91.6) | 30 (85.7) | 419 (94.6) | 98 (97.0) | 359 (93.0) | 457 (93.8) | 438 (93.0) |
| CT or TT | 10 (4.0) | 20* (8.4) | 5 (14.3) | 24† (5.4) | 3 (3.0) | 27 (7.0) | 30 (6.2) | 33 (7.0) |
| Allele | | | | | | | | |
| C | 486 (98.0) | 457‡ (95.6) | 65 (92.9) | 861§ (97.2) | 199 (98.5) | 744 (96.4) | 943 (96.8) | 909 (96.5) |
| T | 10 (2.0) | 21 (4.4) | 5 (7.1) | 25 (2.8) | 3 (1.5) | 28 (3.6) | 31 (3.2) | 33 (3.5) |

*ApoE4 (+) vs. ApoE4 (−): $df = 1$, $\chi^2 = 3.96$, $p = 0.0466$. †Family history (+) vs. family history (−): $df = 1$, $\chi^2 = 4.48$, $p = 0.0344$. ‡ApoE4 (+) vs. ApoE4 (−): $df = 1$, $\chi^2 = 4.46$, $p = 0.0346$. §Family history (+) vs. family history (−): $df = 1$, $\chi^2 = 3.96$, $p = 0.0459$.

## Discussion

This study showed that (1) the C270T and G196A BDNF gene polymorphisms are in incomplete disequilibrium. (2) The frequencies of the GG and GA genotypes and the G allele of the G196A polymorphism were significantly higher in our AD subjects than in our control subjects. (3) The distribution of the C270T polymorphism did not differ significantly between AD and control subjects. (4) The frequency of the T allele of the C270T polymorphism was significantly higher in both ApoE4-positive and family history-negative AD.

None of the known previous studies of the G196A and C270T polymorphisms of the BDNF gene examined both simultaneously. In the first of the three studies, Kunugi et al. (2001) found that the frequency of the T allele of the C270T polymorphism (7.6%) was significantly higher in late-onset AD cases than in controls. The 2.1% frequency of the T allele in their control subjects (Kunugi et al., 2001) is comparable with the frequency of the T allele in our controls (3.5%).

In the second study, examination of the association between C270T polymorphism and AD by Riemenschneider et al. (2002) showed a significantly higher frequency of the T allele in AD cases in general (6.2%) than in controls. This association was more prevalent in AD patients lacking ApoE4, especially in early-onset cases. We could not replicate the Riemenschneider study's overall association between AD and the C270T polymorphism, nor could we find the reported effect of the T allele on the onset of disease in our AD subjects. (The mean age at onset for our cases with the CC genotype was $70.4 \pm 8.5$ years; for those with the CT genotype, it was $72.0 \pm 9.2$ years.) Nevertheless, our results do support the C270T association with AD in subjects lacking ApoE4. Although we found a higher frequency of the T allele in AD cases with family history of dementia, only a few of our cases had AD in their family history. Therefore, these results remain to be confirmed in a larger sample.

Only the third of the three previous studies examined the association between the G196A polymorphism and AD. Ventriglia et al. (2002) showed a significantly higher frequency of the GG genotype of the G196A polymorphism in AD subjects than in controls, regardless of ApoE4 status. There was a nonsignificant trend toward a higher frequency of the G allele of the G196A polymorphism in cases than in controls (Ventriglia et al., 2002). The results of our study using a much larger sample support these findings.

There are three potential explanations for the (relatively weak) association we found: First, a recent study by Egan et al. (2003) suggested that the G196A polymorphism is functional, and that this polymorphism affects intracellular distribution, packaging, and release of the BDNF protein in vitro. They reported that the polymorphism had significant effects on verbal episodic memory, hippocampal activation, and measures of hippocampal neuronal integrity and synaptic abundance (Egan et al., 2003). Given the recent finding by Michalski and Fahnestock (2003) that proBDNF protein is lower in the AD parietal cortex, the G196A polymorphism might play some role in the development of AD, by altering the expression of proBDNF. However, using functional magnetic resonance imaging (fMRI) and $^{1}H$ magnetic resonance spectroscopic imaging

(MRSI), Egan and colleagues showed that the A allele of the G196A polymorphism was associated with poorer episodic memory, abnormal hippocampal activation, and lower hippocampal N-acetyl aspartate levels (NAA) in healthy subjects. Our results suggesting association of the G allele of the G196A polymorphism with AD are inconsistent with those findings. Moreover, because the results of the Egan study were obtained from healthy subjects, those study findings do not explain the pathophysiology of AD. Thus, the role of the G196A polymorphism in the pathophysiology of AD remains unknown.

The second possible explanation is that this polymorphism may be in linkage disequilibrium with other polymorphisms elsewhere in the gene, demonstrating biologically relevant variability. This seems unlikely, however, inasmuch as an extensive search has failed to identify common polymorphisms accompanying amino acid replacement in this gene (Egan et al., 2003; Weese-Mayer et al., 2002). Finally, the third explanation is the possibility that this polymorphism is in linkage disequilibrium with a genetic variation of another gene located near the BDNF gene.

Although with sufficient statistical power we found a positive association between BDNF gene polymorphisms and AD, our findings need to be replicated in still larger, independent samples, or in family-based samples, to reach firm conclusions.

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# The entorhinal cortex regulates blood glucose level in response to microinjection of neostigmine into the hippocampus

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## Abstract

**OBJECTIVE**: Microinjectin of neostigmine, an inhibitor of acetylcholine esterase, into the rat hippocampus elicited stress-like responses reflected by the release of adrenocorticotropic hormone (ACTH) and blood glucose elevations. The entorhinal cortex is regarded as an interface between the hippocampus and neocortex. The current study was designed to examine the role of the entorhinal cortex in regulation of blood glucose elevation induced by hippocampal neostigmine injection.

**MARERIAL AND METHODS**: We produced the entorhinal cortex lesions in 9 week-old male Wistar rats by the bilateral injections of the cell-selective neurotoxin, ibotenic acid (15 μg / μl). Two weeks after the injections, neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected into the rat hippocampus in a volume of 1 μl for 1 min using a CMA/100 microinjection pump.Plasma ACTH levels were measured by radioimmunoassay. Plasma glucose concentrations were determined by the immobilized enzyme membrane/$H_2O_2$ method with a compact glucose analyzer Antsense II (Bayer Medical Co.Ltd,Tokyo, Japan).

**RESULTS**: Compared with sham-operated control rats, the entorhinal lesions produced by ibotenic acid significantly attenuated the elevations of blood glucose evoked by the microinjection of neostigmine into the hippocampus. However, no significant difference of plasma ACTH in response to the injection was observed between the entorhinal-lesioned rats and controls.

**CONCLUSION**: The results of the present study indicate that the entorhinal cortex plays a role in the central nervous systems regulation of blood glucose and may be involved in a stress response presumably via an alternative pathway.

ORIGINAL ARTICLE

**Abbreviations:**

| | |
|---|---|
| ACTH | adrenocorticotropic hormone |
| EC | entorhinal cortex |
| CNS | central nervous systems |
| CRH | corticotropin releasing hormon |
| PVN | paraventricular nucleus |
| HPA | hypothalamic-pituitary-adrenal |
| BNST | bed nucleus of the stria terminalis |

## Introduction

Stress is common to all living creatures regardless of differences in its quality or intensity. The imposition or perception of environmental or physical change, negative or positive, elicits a spectrum of physiologic changes that can be construed as adaptive to the organism. Prominent among these is the release of glucocorticoids by the adrenal glands, which serves to alert the organism to environmental or physiologic changes and to preserve homeostasis. Levine and Ursin [10] provided a definition of stress that consists of three elements: stimulus input, central processing system, and response output; with biological and psychological processes viewed as integral parts of the general homeostatic principle. The brain perceives inputs of various stressors and responds via the nervous, endocrine and immune systems, which are called stress responses [17]. In this sense, the brain plays a role in governing the stress responses. Elevations of corticotropin-releasing factor, ACTH and glucocorticoids are the main features of reactions to diverse and acute stressful stimuli [2, 24]. During stress, neurons of the hypothalamic paraventricular nucleus (PVN) release corticotropin-releasing hormone into the pituitary portal circulation, and ACTH secreted from the anterior pituitary gland in response to corticotropin-releasing hormone, stimulates the secretion of glucocorticoids from the adrenal gland. This constitutes the hypothalamic-pituitary-adrenal (HPA) axis, which is the major regulator of neuroendocrine stress responses [1,5,7,11,13,15]. Involvement of the limbic system in neuro-endocrine responses to some stressors has been documented. A wealth of evidence suggests that the hippocampal cholinergic system is involved in some stress responses [9,12,21]. In particular, the cholinergic system in the hippocampus plays a role in regulating the peripheral metabolism of glucose and catecholamines [7,21]. Under stress, the release of acetylcholine in the hippocampus increases, which coincides with the elevation of plasma glucose and catecholamines [19]. In our previous experiments, we observed that the administration of neostigmine, an acetylcholine esterase inhibitor, into the hippocampus elevates the levels of blood glucose and ACTH. Thus, we concluded that the microinjection of neostigmine into the hippocampus is a potential experimental model for acute stress responses [7,8]. The entorhinal cortex is a gateway to the hippocampus. Many sensory inputs and other information reach the hippocampus via the entorhinal cortex. It receives inputs from the neocortex, including the temporal and frontal lobes, amygdala and olfactory bulbs [3]. Information enters the hippocampal formation via the entorhinal cortex and exits via the fornix. Also, the entorhinal cortex is the primary supplier of converging neocortical sensory input to the ipsilateral dentate gyrus of the hippocampal formation [20]. We previously reported on the involvement of the entorhinal cortex in the stress response to immobilization [22]. Lesions in this area produced by ibotenic acid attenuate ACTH elevation during immobilization stress but not during insulin-induced hypoglycemia. The aim of this study was to investigate the role of the entorhinal cortex in stress responses. We produced bilateral entorhinal lesions using ibotenic acid in rats, and observed the peripheral responses of stress markers induced by microinjections of neostigmne into the hippocampus.

## Material and Methods

*Subjects:* We used 9 week-old male Wistar rats (200–300 g) for the experiment. The animals were individually housed under standard laboratory conditions in temperature-controlled rooms (25 °C), and were maintained under a 12 h light/dark cycle (light on at 06.00) with food pellets and water available *ad libitum*. The rats were cared for in accordance with the ethical guidelines approved by the Animal care and Use Committee of Nagoya University.

*Experimental protocol:* Rats were randomly assigned to one of two major groups: unlesioned or lesioned.

The rats in each group were then divided into two subgroups: Group 1: unlesioned neostigmine-injected, Group 2: unlesioned saline-injected, Group 3: lesioned neostigmine-injected, and Group 4: sham-operated rats, neostigmine-injected.

*Surgery:* The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and mounted in a stereotaxic frame (Narishige Scientific Instruments Laboratory, Tokyo, Japan). For insertion of the stainless steel needle, the skull was exposed and a burr hole was drilled overlying the injection coordinates. Ibotenic acid was injected through a stainless steel needle (outside tip diameter of 28 μm), which was connected to a 1.0 μl syringe via a 30 cm tube filled with the injection solution. Coordinates for the entorhinal cortex were calculated relative to Bregma with the incisor bar set at –3.30 mm. The coordinates used were anterior-posterior –6.04 mm, medial-lateral ±6.50 mm and dorsal-ventral 7.00 mm from the skull surface in accordance with the atlas of Paxinos and Watson [14]. Entorhinal cortex lesions were produced by pressure-injection of 0.1 μl of ibotenic acid (15 μg / μl in 0.9% NaCl, Sigma Chemical Co., St Louis, MO, USA) bilaterally over 5 min. The tip was allowed to remain in the brain for 5 min after injection to minimize dorsal diffusion of the drug along the needle tract. Sham-operated rats were treated in an identical manner to the ibotenic acid-lesioned rats but were injected with the same volume of saline without ibotenic acid.

A recovery period of 7 days was given to the above operated rats (Group 3, 4), otherwise all rats were anesthetized one week before the experiment to stereotaxically implant a guide cannula (Bas, Tokyo, Japan) into the left dorsal hippocampus at the following coordinates: anterior-posterior –2.0 mm, medial-lateral 1.5 mm, dorsal-ventral 3.5 mm in accordance with the Paxinos and Watson atlas [14] one week before the experiments.

The day before the experiments, the rats were anesthetized with diethyl ether (Kanto Chemical Co. Inc, Tokyo, Japan), and a catheter was inserted into the jugular vein for repeated blood sampling. A 2 cm longitudinal incision was made in the neck directly over the trachea. The underlying muscles were separated using blunt dissection and the right jugular vein was catheterized with Silastic tubing (Shiniest Polymer, Nagoya, Japan) filled with heparinized saline. The catheter was threaded through the vein over a distance of 2.5 cm, which allowed the tip of the cannula to rest in or near the atrium. The free end of the catheter was plugged with a knot and the catheter exteriorized and secured at the back of the neck with a special cap.The rats were kept in individual cages with free access to water and food.

*Procedures:* Two weeks after developing entorhinal cortex lesions, saline containing neostigmine methylsulfate (sigma, $5x10^{-8}$ mol) was microinjected in a volume of 1µl for 1 min using a CMA /100 microinjection pump (BSA, Tokyo, Japan) through the guide cannula into the left dorsal hippocampus of free moving rats. To determine the plasma concentration of ACTH and glucose, blood was intermittently sampled (0.8 ml), starting at time 0, just before injection, and at 10, 30, 60 and 120 min after. To minimize the effect of volume loss, an equal volume of heparinized saline was retuned to the general circulation at each sampling. The blood samples were kept on ice, centrifuged, and the plasma was removed and stored at –20 °C in 400 µl aliquots for subsequent determination of ACTH by radioimmunoassay [16]. Plasma glucose concentrations were determined by the immobilized enzyme membraine/$H_2O_2$ method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) (21). All experiments were completed between 10.00 h and 13.00 h to minimize variability resulting from circadian rhythm. Two hours' after neostigmine injection, the rats were deeply anesthetized with a lethal dose of sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). They were postfixed over night and cryoprotected in phosphate-buffered saline containing 30% sucrose for 2 days.

*Histological verification:* For the verification of lesions and the effect of the vehicle, the postfixed brains were frozen with powdered dry ice and serial sections 20 µm in thickness were processed from the region of the entorhinal cortex and mounted on glass slides. Selected regions were stained with Cresyl Violet to assess the extent of the lesions in the entorhinal cortex.

Data and statistical analysis: All sections were assessed by means of microscopic examination using an Olympus BX50 microscope (Tokyo, Japan). Photographs were made of representative lesions and vehicle injection sites in the entorhinal cortex. Blood glucose concentrations were expressed as means ±S.E.M, and differences between the four experimental groups were assessed using repeated measures of one factor ANOVA. Plasma ACTH levels were measured by radioimmunoassay [16], and plasma glucose concentrations were determined by the immobilized enzyme membrane/H2O2 method with a compact glucose Antsense II analyzer (Bayer Medical Co. Ltd, Tokyo, Japan) [18].

## Results

Figure 1 shows Nissle staining of representative sections including the entorhinal cortex. The significant loss of neurons accompanied by extensive glial proliferation was observed in the entorhinal cortex sections of animals that received ibotenic acid injections (Group 3) (Fig. 1D,E). Animals that received injections of vehicle in the entorhinal cortex did not show any histological signs of neuronal damage (Fig. 1B,C). Figure 2 shows the ACTH and blood glucose concentrations for the lesoined and unlesioned rats following microinjectin of neostigmine into the hippocampus. Figure 2A shows the blood glucose concentration after microinjection of neostigmine into the hippocampus. For Group 1, the plasma concentration of glucose increased after 10 min and reached a peak after 60 min. The saline injected group (Group 2) showed no effect. For the lesioned groups, blood glucose levels for Group 3 were significantly lower than those of Group 4 (Fig. 2A). ANOVA showed that there was a statistically significant difference among the groups ($p<0.0001$), and Scheffe's post–hoc analysis indicated that ibotenic acid lesions significantly attenuated blood glucose release evoked by the microinjection of neostigmine into the hippocampus (Fig. 2A). No significant difference was observed in the plasma ACTH concentration between Group 1, 3 and 4 after the microinjection of neostigmine into the hippocampus (Fig. 2B).

## Discussion

In the current study, we have discovered that the entorhinal cortex is involved in the regulation of stress-like responses induced by hippocampal neostigmine injection. The lesions in this area significantly attenuated the blood glucose elevation but did not affect ACTH secretion. No significant difference of weight was observed before and after lesion.

During stress, an adaptive or compensatory response by the organism is activated to sustain homeostasis. Stress induces adaptation through the produc-