Decreased levels of BDNF protein in Alzheimer temporal cortex are independent of BDNF polymorphisms

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Received 27 August 2004; revised 24 December 2004; accepted 19 January 2005

Abstract

Levels of brain-derived neurotrophic factor (BDNF) are reduced in specific brain regions in Alzheimer’s disease (AD) and BDNF gene polymorphisms have been suggested to influence AD risk, hippocampal function, and memory. We investigated whether the polymorphisms at the BDNF 196 and 270 loci were associated with AD in a clinical and neuropathological cohort of 116 AD cases and 77 control subjects. To determine how BDNF protein levels relate to BDNF polymorphisms and AD pathology, we also measured BDNF in temporal association cortex, frontal association cortex, and cerebellum in 57 of the AD and 21 control cases. BDNF protein levels in temporal neocortex of the AD brains were reduced by 33% compared to control brains, whereas levels were unchanged in frontal and cerebellar cortex. The BDNF genotypes were not significantly associated with a diagnosis of AD, although the BDNF 270 C allele was slightly overrepresented among carriers of the APOE ε4 allele. Moreover, BDNF protein levels did not differ between the various BDNF genotypes and alleles. Neuropathologically, the loss of BDNF in AD showed a weak correlation with accumulation of neuritic amyloid plaques and loss of the neuronal/synaptic marker synaptophysin. The results suggest that the investigated BDNF polymorphisms are neither robust genetic risk factors nor determinants of BDNF protein levels in AD.

Keywords: BDNF; Alzheimer’s disease; ELISA; Gene polymorphisms; Association studies; Synaptophysin

Introduction

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is essential for neuronal survival both in the peripheral and central nervous system (CNS) (Murer et al., 2001). In CNS, BDNF is enriched in the hippocampal formation, cerebral cortex, and the amygdaloid complex (Murer et al., 2001)—regions that are widely affected by deposition of amyloid plaques and neurofibrillary tangles (NFTs) in Alzheimer’s disease (AD) (Ingelsson and Hyman, 2002). In the AD brain, decreased BDNF protein levels were reported in hippocampus, entorhinal cortex, and temporal neocortex (Hock et al., 2000) while no changes were observed in areas less affected by the disease, such as the frontal, parietal, and cerebellar cortices (Hock et al., 2000; Narisawa-Saito et al., 1996). In addition, BDNF mRNA levels were found to be substantially decreased in human AD parietal cortex (Holsinger et al., 2000).

The human BDNF gene is located on chromosome 11 (Maisonpierre et al., 1991) and consists of 13 exons. The entire open reading frame is within the last exon and, due to alternative splicing, encodes two BDNF protein variants (GenBank accession no. AF411339). The long form of BDNF, with 247 amino acids, consists of a 5′ pro-BDNF sequence that is proteolytically cleaved to form the mature protein (Seidah et al., 1996) whereas the short BDNF form, with 153 amino acids, lacks the 5′ pro-region.

Studies of a single nucleotide polymorphism (SNP) in the 5′ pro-region of the BDNF gene implicated the BDNF 196 A

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0014-4886/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2005.01.026
allele to be associated with increased risk for both AD (Ventriglia et al., 2002) and bipolar disorder (Sklar et al., 2002). However, other studies failed to find an association of this BDNF SNP with AD (Combarros et al., 2004; Nishimura et al., 2004) or various psychiatric illnesses (Hong et al., 2003a,b; Lam et al., 2004; Tsai et al., 2003). Nevertheless, subjects possessing the BDNF 196 A allele had poorer episodic memory and hippocampal activation by functional MRI (relative to BDNF 196 GG homozygotes) (Egan et al., 2003, Hariri et al., 2003) and BDNF 196 GA carriers had a significantly lower mean performance IQ than subjects with the BDNF 196 GG genotype in a cohort of young Chinese females (Tsai et al., 2004). Another SNP in BDNF exon 7 showed an association between the BDNF T allele and AD in two different Japanese populations (Kunugi et al., 2001; Nakata et al., 2003) as well as in a German cohort (Riemenschneider et al., 2002).

It is unknown how the various polymorphic BDNF alleles exert their differential influence on brain function and disease processes although cell-culture studies suggest that an impaired secretion of the BDNF isoform encoded by the 196 A allele may mediate some of the observed clinical associations (Chen et al., 2004; Egan et al., 2003). Apart from detecting loss of BDNF in areas of brain affected by AD, the relation of BDNF protein to BDNF polymorphisms and to other biochemical and pathological changes in the AD brain has not been studied.

We investigated AD-related changes in BDNF protein levels with BDNF polymorphisms and AD pathology in a well-characterized pathological cohort. In particular, we hypothesized that the BDNF 196 A allele would be associated with decreased BDNF protein levels in affected brain regions. We also predicted that decreased levels of BDNF would be associated with a more severe disease state as indicated by clinical severity and neuropathological alterations such as synaptic loss, deposition of amyloid plaques, and formation of neurofibrillary tangles.

Materials and methods

Materials

A total of 116 AD and 77 control subjects was included for BDNF genotyping. Pathological specimens were investigated from 56 of the AD and 22 of the control individuals. In the AD group, the mean age was 80.4 ± 0.8 years and 32.8% were male. For control subjects, the mean age was 75.0 ± 1.0 years and 45.4% were male. All AD cases were obtained from the Alzheimer’s Disease Research Center (ADRC) at Massachusetts General Hospital or from the Harvard Brain Tissue Resource Center (grant no. 1R24MH68855) and were neuropathologically diagnosed according to the NINCDS-ADRDA and Reagan criteria (Hyman and Trojanowski, 1997). Twenty-two of the control subjects were autopsied and did not demonstrate any pathology of a neurodegenerative disorder. The remaining 55 clinical control subjects were either non-affected spouses of AD patients or healthy volunteers. Of the AD brains, 106 were genotyped for apolipoprotein E (APOE) polymorphisms and showed an APOEε4 allele frequency of 0.38; the corresponding frequency for the 74 genotyped control subjects was 0.10. The average PMI for the AD cases was 13.4 ± 0.8 and for controls 18.5 ± 3.2. No correlation was found between PMI and the BDNF levels.

Brain dissection and sample preparation

For BDNF protein measurement, strips of gray matter from temporal, frontal, and cerebellar cortices were dissected from fresh frozen brain tissue, with care taken to avoid underlying white matter, and homogenized in the following extraction buffer: 50 mM Tris, pH 7.2, 0.1% Triton X-100, 2 mM NaCl, 2% protease inhibitors (Complete, Roche, Indianapolis, IN) and 2% protease free bovine serum albumin (BSA; Sigma, St. Louis, MO) (40 µl/mg tissue). The homogenate was centrifuged at 15,000 rpm (5 min) and the resulting supernatant was used for the BDNF ELISA.

For genetic analyses, 50–150 mg of brain tissue was dissected from each autopsied subject and the QIAamp DNA Mini Kit (QIAGEN, Los Angeles, CA) was used for DNA extraction according to the manufacturer’s instructions. In brief, the tissue was homogenized, treated with Proteinase K and incubated at 56°C overnight. Next, the samples were centrifuged at 8000 rpm for absorbance through a QIAamp spin column, removed of any residual contaminants by washing with two centrifugation steps and finally eluted from the QIAamp spin column. For the clinical cases, DNA was extracted from whole blood samples, also by using the QIAamp DNA Blood Mini Kit (QIAGEN). These blood samples were subjected to the same protocol as described above, but with the incubation at 56°C for 10 min. Finally, the DNA was stored at −20°C until genotyping.

ELISA

The BDNF ELISA utilized MAB848 (monoclonal mouse anti-BDNF, R&D Systems, Minneapolis, MN) as capture antibody and biotinylated BAM648 (R&D Systems) as detector antibody. Microtiter plates (96-well) (Greiner, Longwood, FL) were coated with MAB848 (75 µl/well) of 0.5 µg/ml in carbonate buffer, 100 mM, pH 9.6) at 4°C overnight. The plates were washed three times with phosphate buffered saline (PBS), pH 7.0 followed by blocking with 25% BlockAce (Dai-Nippon, Osaka, Japan). The samples and BDNF standard (100 µl/well) were added to the wells containing 25 µl of the extraction buffer and incubated overnight at 4°C. Plates were washed four times with Triton wash buffer (50 mM Tris, pH 7.2, 0.1% Triton X-100, 200 mM NaCl, 2 mM EDTA) and then incubated at...
room temperature (2 h) with biotinylated BAM648 (75 μl/ well, 1:1000 in extraction buffer). The plates were washed four times with Triton wash buffer followed by incubation with horseradish peroxidase-linked streptavidin (75 μl/well, 1:1000 in extraction buffer, Jackson, West Grove, PA) at room temperature (2 h). After washing six times with Triton wash buffer, fluorometric measurements using 320 nm excitation filter and 400 nm emission filter were obtained after addition of QuantaBlu substrate (Pierce, Rockford, IL). Increasing dilutions of recombinant human BDNF (Chemicon, Temecula, CA) in Triton extraction buffer was used as a standard for every plate; a best fit log–log curve, at dilutions below saturation, was utilized as the standard curve.

Genotyping

Genotyping of the BDNF 196 polymorphism was performed according to a previously published protocol (Nakata et al., 2003), but with 10% dimethyl-sulfoxide (DMSO) omitted and the annealing temperature set at 62°C. The 171 bps PCR-product was subjected to digestion by restriction fragment length polymorphism (RFLP) at 37°C by PmlI (12 h), yielding either the non-cleaved 171 bps fragment (AA), a triplet of 72, 99, and 171 bps fragments (AG) or a doublet of 72 and 99 bps fragments (GG) (Fig. 1A).

In order to verify the activity of PmlI for AA homozygotes (where no cleavage occurs), a separate PCR-product (including the 196 polymorphic site) amplified from a GG homozygote, was added to the restriction cleavage reaction of all samples. This 574 bps product yielded, after digestion, two fragments of 133 bps and 441 bps. The PCR primers used to generate this internal control were 5' GAA AGA AGC AAA CAT CCG AGC ACG TG 3' (fwd) and 5' CTG GGT AGT TCG GCA CTG 3' (rev). The PCR was carried out in a total volume of 60 μl containing 5.0 μl of 10× PCR reaction buffer (Roche) with 1.5 mM Mg, 1.0 U of Taq DNA polymerase (Roche), 10 pmol of each primer, 10 nmol of dNTPs, and 10 μl DNA. The reaction was initiated at 95°C for 5 min, followed by 35 amplification cycles (denaturation at 95°C for 1 min, annealing at 55°C for 30 s, extension at 72°C for 1 min) and a final extension step of 10 min at 72°C.

The BDNF 270 polymorphism was also genotyped according to a previously published method (Nakata et al., 2003), but with 10% dimethyl-sulfoxide (DMSO) omitted and the annealing temperature set at 60°C. RFLP was performed by subjecting the 233 bps PCR-product to cleavage by the HinfI restriction enzyme. The samples were digested at 37°C (6 h), followed by heat-inactivation at 80°C (20 min). After digestion, the following combinations of fragments were produced: 18, 78, and 127 bps (CC); 15, 18, 63, 78, and 127 bps (CT). No TT genotypes were identified (Fig. 1B).

All digested samples were analyzed by microcapillary gel electrophoresis, using the Agilent 2100 Bioanalyzer (DNA 500 LabChip, Agilent Technologies, Palo Alto, CA).

Other biochemical and neuropathological analyses

Biochemical assessments of amyloid-β (Aβ) 40, Aβ42, synaptophysin, and glial fibrillary acidic protein (GFAP) as well as neuropathological analyses of diffuse and neuritic amyloid plaques and neurofibrillary tangles (NFTs) were performed on 78 of the included AD and control brains. These brains were all part of a recently well-characterized cohort (Ingelsson et al., 2004).

Statistical analyses

The primary analyses were BDNF levels and BDNF allelic polymorphisms in AD vs. control subjects. Significant differences in the primary analyses were followed up by post hoc exploratory analyses of BDNF levels and pathological measures. The latter were not corrected for multiple comparisons.

An unpaired t test (Statview, SAS, Cary, NC) was used to analyze the differences in BDNF protein levels between AD and control groups. The genotype and allele frequencies for AD and control cases were evaluated by a chi-square test (Statview). Post hoc simple regression analyses were performed in order to seek for correlations between BDNF...
protein levels and levels of the various biochemical and neuropathological markers. Sample size analyses (Stata, College Station, TX) demonstrated that there was 0.80 power to test a hypothetical difference of 15% in the frequency of the more prevalent BDNF 196 G allele and 0.86 power to detect the previously reported increase in BDNF 270 T carriersonship in AD (Kunugi et al., 2001). All results are given as mean levels ±1 standard error (SE).

Results and discussion

ELISA measurements demonstrated that BDNF protein levels in the temporal neocortex were reduced by 33% in AD relative to control brains (P < 0.0001), while no differences in BDNF levels were observed in frontal or cerebellar cortex from AD and control brains, respectively (Table 1). Levels of BDNF were not associated with duration of illness of AD, a surrogate marker for disease severity (not shown).

The distribution of alleles for both of the BDNF SNPs investigated were in Hardy–Weinberg equilibrium. The genotype/allele frequencies for neither the BDNF 196 (genotype: P = 0.10; allele: P = 0.33) nor the BDNF 270 (genotype: P = 0.11; allele: P = 0.12) polymorphisms were significantly different between AD and controls (Table 2). When we divided the subjects according to their respective APOE genotypes, the tendency for increased disease risk seen with the BDNF 270 C allele became weakly statistically significant among APOE e4 carriers only (genotype: P = 0.04; allele: P = 0.05, data not shown). For the BDNF 196 SNP, APOE genotypic status did not influence the distribution of BDNF genotypes or alleles among AD and control subjects. Finally, neither the BDNF nor the APOE polymorphisms significantly affected the age of onset in the AD group.

No statistically significant effects were observed when comparing the BDNF protein levels in the various brain regions to the different BDNF genotypes and alleles, regardless of whether the analysis was performed on the entire cohort or on the AD and C groups separately. We also analyzed whether the various APOE genotypes were correlated with BDNF protein levels and found slightly decreased levels in the temporal cortex of APOEe4 carriers (P = 0.04, data not shown). This effect is most likely explained by the overrepresentation of AD cases (with lower BDNF levels) among APOEe4 carriers; when analyzing AD brains alone, no difference in BDNF levels was seen between cases with and without the APOEe4 allele.

To explore the pathological correlates of the decreased BDNF levels in AD, we performed simple regression analyses between temporal BDNF levels and the various biochemical and neuropathological brain measures. The levels of synaptophysin were associated with BDNF protein levels in the temporal neocortex (P = 0.05). The burden of neuritic amyloid plaques was inversely correlated with temporal BDNF protein levels (P = 0.02) (Fig. 2).

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<td><strong>BDNF levels in temporal, frontal, and cerebellar cortex of AD and control brains</strong></td>
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<td>Brain area</td>
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Table 2

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<th>Genotype and allele frequencies of the BDNF 196 and 270 polymorphisms</th>
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<tr>
<td><strong>BDNF 196</strong></td>
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<td>AD (n = 95)</td>
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<td>Control (n = 70)</td>
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<tr>
<td><strong>BDNF 270</strong></td>
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<tr>
<td>AD (n = 106)</td>
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<td>Control (n = 73)</td>
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Fig. 2. (A) Decreased levels of temporal BDNF correlate with decreased levels of synaptophysin. (B) Decreased levels of temporal BDNF correlate with higher load of neuritic amyloid plaques, as stained by ThioS. *P < 0.05.
statistically significant correlations between BDNF protein levels and levels of Aβ40, Aβ42, GFAP, diffuse amyloid plaques, or NFTs were observed. Since several significance tests were performed, the correlations obtained have to be interpreted with caution.

In summary, we find that diagnosis of AD and neuro-pathologic severity were not associated with polymorphisms at the 196 and 270 loci of BDNF. The regional loss of BDNF in temporal cortex, the earliest and most severely affected part of the AD brain, over frontal cortex and cerebellum supports previous findings (Connor et al., 1997; Hock et al., 2000; Narisawa-Saito et al., 1996). Moreover, the relationship of BDNF levels with synaptic loss and neuritic plaques are consistent with the hypothesis that the decline in BDNF, a neuronal protein, is a reflection of the overall neuronal/synaptic loss and plaque-associated neuritic dystrophy. The loss of such a potent neurotrophic factor for cortical neurons could also increase the susceptibility of the remaining neurons to AD-related damage, whether due to neuritic plaques, tangle formation, oxidative stress, lipid dysregulation, toxic amyloid species, inflammation, etc.

In contrast with prior studies (Kunugi et al., 2001; Riemenschneider et al., 2002; Ventriglia et al., 2002), AD was not associated with polymorphisms at the BDNF 196 or BDNF 270 loci in our case-control series. Potential explanations for our findings include differences in the populations studied and in case-ascertainment. Positive associations for the BDNF 196 and 270 SNPs were from Italian and German/Japanese populations, respectively, while our cohort was from North America. Moreover, previous association studies were based mainly on clinically diagnosed cases, while all the AD patients and many control subjects included in our study had an autopsy-confirmed diagnosis.

No genotype–phenotype correlation was observed between the BDNF polymorphisms and the BDNF protein levels. Therefore, the principle determinant of BDNF levels in temporal cortex is the AD disease state and severity, rather than the BDNF polymorphisms. Although cell-culture studies found that the BDNF 196 A allele reduced BDNF protein secretion, we did not detect a reduction of total BDNF in the brains of BDNF 196 A allele carriers. Although it would have been reasonable to ask whether BDNF 196 has a differential effect on BDNF mRNA levels, we decided to focus on protein analyses due to the fact that previous cell-culture-based studies suggested the functional effect of BDNF 196 to be linked to BDNF protein and not mRNA levels (Egan, 2003 #6) (Chen, 2004 #58).

In conclusion, our study demonstrated a marked decrease of BDNF protein levels in AD temporal neocortex, which tended to be associated with neuropathological measures of disease severity. Apart from a weak correlation between the 270 C allele and AD only among APOEε4 carriers, AD diagnosis and brain BDNF protein levels were largely independent of reported BDNF polymorphisms, suggesting that the two SNPs are not robust genetic risk factors for AD or the primary determinants of BDNF protein levels in AD. The regional deficit of BDNF in AD may either result in or be a consequence of synaptic loss and fibrillar amyloid deposition in the AD brain and potentially increase the susceptibility of these brain regions to further degeneration.

Acknowledgments

This work was supported by NIH (AG 05134), The Swedish Brain Foundation, The Swedish Research Council, and the AFAR Beeson Award. We are also grateful to Harvard Center for Neurodegeneration and Repair (Harvard Medical School, Boston MA).

References


