Preservation of gray matter volume in multiple sclerosis patients with the Met allele of the rs6265 (Val66Met) SNP of brain-derived neurotrophic factor

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Received June 12, 2007; Revised and Accepted July 12, 2007

To investigate the association of the rs6265 (Val66Met) single nucleotide polymorphism (SNP) of brain-derived neurotrophic factor (BDNF) with brain morphometry and functional status as measured by quantitative magnetic resonance imaging (MRI) and neurocognitive testing in multiple sclerosis (MS) patients. BDNF is released by neurons and by immune cells in MS brain. The rs6265 SNP variation of BDNF causes substitution of valine (Val) for methionine (Met) and interferes with activity-dependent BDNF secretion. A total of 209 treated MS patients (161 females; 48 males) underwent clinical brain MRI and were genotyped for the BDNF rs6265 Val66Met SNP. A subset of 108 patients had neurocognitive testing for processing speed, memory and executive function. The MRI measurements included T2 and T1-lesion volume (LV); normalized brain volume measures of whole brain (WB) volume, white and gray matter volume (NWMV and NGMV) and the diffusion-weighted imaging measure of WB mean parenchyma diffusivity (MPD). The Met66 allele status was positively associated with NGMV (\(P=0.015\), standardized \(\beta=0.15\)) and negatively associated with T2-LV (\(P=0.041\), standardized \(\beta=-0.14\)). There were no significant associations between Met66 allele status and T1-LV, NWMV or MPD. On the Paced Serial Addition Test (PASAT), a trend (\(P=0.057\)) favoring the Met66 allele group was observed. There were no significant associations between Met66 allele status and other neurocognitive measures. The BDNF Met66 allele is associated with lower damage as evidenced by measurement of NGMV and T2-LV in MS patients.

INTRODUCTION

Multiple sclerosis (MS) is a degenerative, chronic inflammatory disease of the central nervous system (CNS) that causes demyelination, sclerotic plaque formation and CNS atrophy. The etiology and pathogenesis of MS remain poorly understood despite considerable evidence suggesting that CNS damage in MS is the result of abnormal immune responses against the patient’s nervous tissue. However, recent histopathological findings have demonstrated that MS is also associated with a significant neurodegenerative component and progressive neuronal loss secondary to the initial inflammatory process (1–3).

Brain-derived neurotrophic factor (BDNF) is a homodimeric neurotrophic factor that can promote neuronal growth and repair. BDNF is abundantly expressed in the adult brain and it is also produced by immune cells. In the brain, BDNF is transported and released by neurons where it plays key roles in synaptic plasticity.

Multiple sequence variations in BDNF gene have been described, including single nucleotide polymorphisms (SNPs) and more recently, the occurrence of alternatively spliced mRNAs that may lead to variations in gene expression or protein metabolism causing selective neuronal vulnerability (4,5). The genetic variation that is the focus of this paper is a G-to-A substitution (dbSNP identifier: rs6265) in the
**BDNF** gene, which results in replacement of the valine (Val) at codon 66 of the BDNF pro-protein by methionine (Met). In the US population, the frequency of the G/G, G/A and A/A genotypes of rs6265 are 70, 25 and 5%, respectively (6). It is commonly referred to as the Val66Met polymorphism of BDNF and this genetic variation has been shown to strongly affect BDNF functions. Individuals who are G/G homozygous produce only the valine containing isoform of BDNF protein, A/A homozygous individuals produce only the methionine containing isoform of BDNF protein and G/A heterozygous individuals produce both isoforms. The Val66Met polymorphism has been shown to interfere with the intracellular trafficking and the activity-dependent secretion of BDNF (6,7). The trafficking of BDNF in axons is important to its functions in the CNS because BDNF must be transported in the anterograde and retrograde directions to exert its full spectrum of neuronal signaling activities in the CNS. Egan et al. (6) demonstrated that the homodimeric Val66–Val66 isoform of BDNF produced in cells transfectected with only the Val66 BDNF isoform construct localized to secretory granules and synapses more efficiently than the heterodimeric Val66–Met66 BDNF resulting from the co-transfection of Val66 and Met66 BDNF isoform constructs. In vitro experiments in cortical neurons and neurosecretory cells transfected to co-express both the Val66 and Met66 isoforms have demonstrated that 70% of the BDNF consists of Val66–Met66 heterodimers and that the heterodimers are secretion-impaired relative to the Val66–Val66 homodimers (7). These experiments indicate that Val66Met and Met66Met individuals are likely to have dysregulated activity-dependent BDNF secretion in the brain.

BDNF is expressed abundantly throughout the human brain, particularly in areas such as the prefrontal cortex (8) and the impact of dysregulated or diminished BDNF secretion due to the rs6265 Val66Met polymorphism has been documented in healthy subjects and also in patients with neurological or psychiatric diseases (8–11). Pezawas et al. (12) reported bilateral reductions of hippocampal gray matter volumes (GMVs) (right: \( P = 0.001; \ t = 3.41; \) left: \( P = 0.013; \ t = 2.24 \)) in healthy subjects with the Met66 allele compared with Val66Val subjects. These differences were age- and gender-independent and substantial in terms of volume differences in hippocampal clusters. The subjects with the Met66 allele exhibited additional loci of reduced GM volumes, predominately in the lateral convexity of the frontal lobes with peak values encompassing the dorsolateral prefrontal cortex bilaterally. Egan et al. (6) studied schizophrenic patients, their siblings and healthy controls and found that in the Val66Met group, the levels of N-acetylaspartate in the left hippocampus as measured by magnetic resonance spectroscopy were lower compared with a matched Val66Val group. Associations between the Val66Met polymorphism have been reported but not fully confirmed for the risk of developing affective disorders such as bipolar disorder (13,14) and obsessive–compulsive disorder (15) and in neurodegenerative diseases such as Parkinson’s disease (16–18).

The available data from healthy subjects suggest that individuals with the Met66 allele of BDNF rs6265 could potentially be at a disadvantage in CNS diseases affecting brain function. Furthermore, BDNF and its receptor, TrkB, have also been found in active MS lesions (19–21) and together, these findings provided the mechanistic rationale for investigating the Val66Met polymorphism on magnetic resonance imaging (MRI) and cognitive parameters in MS patients.

**RESULTS**

**Patient characteristics**

The demographic and clinical characteristics of our patient population are summarized in Table 1.

A subset of 108 patients had NP testing. The demographic characteristics such as age and female-to-male ratio (\( P = 1, \) Fisher’s exact test) of the subset with NP were similar to those of the subset without NP testing. The clinical characteristics such as age of onset, treatment duration and Expanded Disability Status Scale (EDSS) were also similar. However, disease duration in the subset with NP testing (14.6 ± 8.9 years) was 2.2 years greater (\( P = 0.044 \)) in an independent samples \( t \)-test) compared with the group without NP testing (12.1 ± 8.4 years). The fraction of subjects with the Met66 allele in the group with NP measures (31.5% Met66 allele) was similar (\( P = 0.67 \), Fisher’s exact test) to that in the group for whom NP was unavailable (34.7% Met66 allele).

The **BDNF rs6265 Val66Met genotype allele distribution is not associated with higher susceptibility for MS**

The rs6265 G-to-A SNP variation that causes the Val66Met substitution in pro-BDNF was genotyped in 209 patients with clinically definite relapsing-remitting MS (RRMS). As summarized in Table 1, 140 (67.0%) patients were Val66Val, 62 (29.7%) were Val66Met and seven (3.3%) were Met66Met. The allele frequency of the G allele (Val66) was calculated to be 81.8% and that of the A allele (Met66) was 18.2%. The genotype frequencies were consistent with Hardy–Weinberg equilibrium. In addition, the allele frequencies obtained in MS patients were compared separately with the frequencies in the cohort of healthy controls reported by Egan et al. (6) and were found to be similar. These similarities suggest that the Val66Met polymorphism does not determine susceptibility to MS.

The **BDNF rs6265 Val66Met genotype is not associated with age of onset of ms or disability**

The age of onset of MS symptoms was similar in patients with the wild type Val66Val and Met66 forms (Val66Met plus Met66Met) of BDNF (31.6 ± 9.4 years in the Val66Val group versus 32.0 ± 9.3 years in the Met66 group; \( P = 0.76 \) in a \( t \)-test).

In regression analyses (\( F = 19.5, \ P < 0.001, \) adjusted \( R^2 = 0.35 \) correcting for gender, presence/absence of progressive MS, age, disease and treatment duration, the EDSS was not associated with the presence (mean ± SD = 3.1 ± 1.8) or absence (mean ± SD = 3.2 ± 1.9) of the Met66 allele (\( P = 0.96 \)). Other groups have reported similar findings (22,23).

The **BDNF rs6265 Val66Met genotype is associated with higher NGMV and lower T2-LV**

The MRI characteristics of the Val66Val and Met66 allele groups are summarized in Table 2.
Table 1. Clinical and demographic characteristics of the cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MRI</th>
<th>MTR subset</th>
<th>NP subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>209</td>
<td>145</td>
<td>108</td>
</tr>
<tr>
<td>Females:males</td>
<td>161:48 (77%)</td>
<td>113:32 (78%)</td>
<td>83:25 (77%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.2 ± 9.1</td>
<td>45.7 ± 8.7</td>
<td>46.2 ± 7.7</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>31.7 ± 9.3</td>
<td>32.3 ± 9.2</td>
<td>31.4 ± 9.5</td>
</tr>
<tr>
<td>Disease duration (years)a</td>
<td>13.4 ± 8.7</td>
<td>13.3 ± 8.5</td>
<td>14.6 ± 8.9</td>
</tr>
<tr>
<td>EDSS</td>
<td>2.5 (1.5–4.0)</td>
<td>2.5 (1.5–4.0)</td>
<td>2.5 (2.0–4.0)</td>
</tr>
<tr>
<td>Disease modifying therapy (years)</td>
<td>5.3 ± 3.4</td>
<td>5.4 ± 3.4</td>
<td>5.5 ± 3.0</td>
</tr>
</tbody>
</table>

Data are mean ± SD, except for EDSS, which is expressed as median (25% quartile–75% quartile range).

Table 2. The cohort’s MRI characteristics. Data are mean ± SD

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients</th>
<th>Val66Val</th>
<th>Met allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of contrast enhancing lesions</td>
<td>0.56 ± 2.0</td>
<td>0.47 ± 1.5</td>
<td>0.73 ± 2.8</td>
</tr>
<tr>
<td>Volume of contrast enhancing lesions (ml)</td>
<td>0.072 ± 0.27</td>
<td>0.057 ± 0.20</td>
<td>0.10 ± 0.37</td>
</tr>
<tr>
<td>T2 LV (ml)</td>
<td>12.4 ± 14.9</td>
<td>13.6 ± 15.3</td>
<td>10.1 ± 14.1</td>
</tr>
<tr>
<td>T1 LV (ml)</td>
<td>2.38 ± 5.1</td>
<td>2.46 ± 4.9</td>
<td>2.23 ± 5.5</td>
</tr>
<tr>
<td>Brain parenchymal volume (ml)</td>
<td>1482 ± 76</td>
<td>1476 ± 77</td>
<td>1494 ± 72</td>
</tr>
<tr>
<td>GMV (ml)</td>
<td>752 ± 66</td>
<td>744 ± 67</td>
<td>766 ± 62</td>
</tr>
<tr>
<td>White matter volume (ml)</td>
<td>730 ± 48</td>
<td>731 ± 49</td>
<td>728 ± 46</td>
</tr>
<tr>
<td>Mean diffusivity</td>
<td>1153 ± 135</td>
<td>1157 ± 132</td>
<td>1142 ± 141</td>
</tr>
</tbody>
</table>

The standard β is the value of the standardized regression coefficients.

The regression results for the MRI parameters are summarized in Table 3. In the regression analysis, there was a positive association between NGMV (F = 12.2, P < 0.001, adjusted R² = 0.24 for final overall model) with the presence of the Met66 allele of BDNF (t = 2.45, P = 0.015, standardized β = 0.15, where the t-statistic is the regression coefficient divided by its standard error, the standardized β is the standardized regression coefficient and the P-value is the observed significance value for the t-statistic testing the hypothesis that the population regression coefficient is zero). This indicates that the presence of the Met66 allele is associated with higher, preserved NGMV in MS patients. The differences between the two groups are shown in Figure 1A. There was a corresponding trend toward positive association between normalized brain volume (NBV) (F = 13.1, P < 0.001, adjusted R² = 0.26 for final overall model) with the presence of the Met66 allele of BDNF (t = 1.82, P = 0.070, standardized β = 0.11). In similar regression analysis, there was a negative association between T2-lesion volume (LV) (F = 4.72, P < 0.001 for final overall model) with the presence of the Met66 allele of BDNF (t = −2.06, P = 0.041, standardized β = −0.14). The T2-LV differences between the two groups are also shown in Figure 1B. This indicates that the presence of the Met66 allele is associated with a trend toward a lower T2-LV in MS patients.

The MTR parameters were available for a subset of 145 patients. The demographic characteristics (Table 1) of the subsets with and without MTR were similar. The mean MTR parameters for the NAGM, normal-appearing brain tissue (NABT) and T2 lesions were not significantly different indicating that the BDNF rs6265 genotype does not alter the processes involved in microscopic damage to GM and brain parenchyma. Next, in order to assess the effect of the BDNF rs6265 genotype on the overall microscopic and macroscopic GM damage, we computed the product of the mean MTR for NAGM and GMV. The product of the mean MTR for NAGM and GMV (F = 4.68, P < 0.001 for final overall model) was positively associated with presence of Met66 allele (t = 2.03, P = 0.045, standardized β = 0.18). There was a trend toward a positive association with the presence of the Met66 allele (t = 1.50, P = 0.14, standardized β = 0.14) for the product of the mean MTR for NABT and NBPV (F = 2.41, P = 0.032 for final overall model). We also conducted regression analysis with GMV as the dependent variable (F = 12.0, P < 0.001 for final overall model) that additionally corrected for MTR of NAGM and found a significant positive independent effect associated with the presence of Met66 allele of BDNF rs6265 (t = 2.72, P = 0.008, standardized β = 0.20).

The associations between the presence of the Met66 allele and T1-LV, NWVM and mean parenchymal diffusivity (MPD) MRI measures and each of the remaining MTR parameters were not significant.

The BDNF rs6265 Val66Met genotype is associated with the PASAT

The NP characteristics of the Val66Val and the Met66 allele groups are summarized in Table 4. All NP parameters are expressed as Z-scores relative to a group of controls evaluated at our Center (24). The P-values for the age-controlled partial
correlations between the NP variable Z-scores and the NGMV are also shown in Table 4. The numerous significant partial correlations underscore the importance of NGMV in determining NP outcomes in MS patients.

A trend toward significant differences favoring the group with the Met66 allele (PASAT Z-score for Val66Val group: \(-0.72 \pm 1.2\) versus PASAT Z-score Met66 allele group: \(-0.32 \pm 1.2\)) was observed (\(t = 1.92, P = 0.057\); standardized \(\beta = 0.21\)) for the PASAT. These PASAT differences between the two groups are shown in Figure 2. No significant differences were seen between the two genotypes in regard to any of the remaining NP variables.

**DISCUSSION**

The degree to which genes and environment determine brain structure and function in normal and diseased states is of fundamental importance. Large-scale neuroimaging and genetic studies are beginning to uncover normal and disease-specific patterns of gene and brain function in large human populations (25). In this report, we have presented data in large cohort of MS patients that demonstrate that the Met66 allele of the rs6265 BDNF SNP is associated with a favorable effect on brain structure and function as measured by MRI and NP testing, respectively.

Consistent with other reports, the rs6265 genotype was not associated with higher susceptibility, lower age of onset or higher disability in MS patients (22,23). The Met66 allele status was shown to have a significant positive association with GM volume (\(P = 0.015\), standardized \(\beta = 0.15\)) and a significant negative association with T2-LV (\(P = 0.041\), standardized \(\beta = -0.14\)). In addition, the product of the mean MTR for NAGM and GMV was positively associated with presence of Met66 allele and there was a trend toward a positive association with the presence of the Met66 allele for the product of the mean MTR of NABT and NBV. These findings were unexpected given the reports indicating that normal individuals with the Met66 allele have poorer episodic memory performance and reduced hippocampal physiologic engagement during memory on functional MRI (6,26). Our findings indicate a new facet of the functional effects of the Val66Met BDNF polymorphism in MS and suggest that the functional associations resulting from the interactions between polymorphisms and disease states, particularly inflammatory/autoimmune diseases [e.g. MS and systemic lupus erythematous (SLE)] can differ from those in healthy individuals.

In brain disorders, increases in brain volume as measured by MRI sometimes may not mean better function because of confounding effects of edema and inflammation, e.g. in MS, both inflammatory processes and axonal loss can mediate WMV changes. However, the principal findings in our study were GMV changes and these were also associated with changes in PASAT performance: the mean PASAT Z-score differences in the Met66 group was 0.4 Z-score units higher than that in the Val66Val group (PASAT Z-score for Val66Val group: \(-0.72 \pm 1.2\) versus PASAT Z-score Met66 allele group: \(-0.32 \pm 1.2\)). Although our study design is cross-sectional and the PASAT is susceptible to practice effects, the mean differences between our genotype groups exceed the 0.15–0.2 Z-score units that were identified by the Polman group as a threshold for evaluating clinical significance of PASAT changes/differences (27). The PASAT differences in our work are statistically medium effect sizes but may be important because they are consistent with and parallel to the changes in the NGMV. It is curious that the PASAT was the only neurocognitive test showing a significant relationship with the rs6265 polymorphism. Previous work has linked the performance of MS patients on the PASAT to global GMV and GMV in regions associated with working memory and executive function (28). This test is heavily reliant on bilateral, dorsolateral prefrontal cortex making connections to posterior association cortices (29,30). In healthy subjects, reduction dorsolateral prefrontal cortex GM is associated with the rs6265
Table 4. The cohort’s NP characteristics

<table>
<thead>
<tr>
<th>Test Z-score</th>
<th>All patients</th>
<th>Met Allele</th>
<th>Partial r</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rs6265 genotype Correlation with GMV*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSFC</td>
<td>0.11 ± 0.70</td>
<td>0.10 ± 0.74</td>
<td>0.35</td>
<td>0.002</td>
</tr>
<tr>
<td>NAART</td>
<td>−0.49 ± 0.97</td>
<td>−0.41 ± 0.86</td>
<td>−0.049</td>
<td>0.64</td>
</tr>
<tr>
<td>CVLT total</td>
<td>−0.70 ± 1.1</td>
<td>−0.61 ± 1.1</td>
<td>0.17</td>
<td>0.086</td>
</tr>
<tr>
<td>CVLT delay</td>
<td>−0.86 ± 1.3</td>
<td>−0.68 ± 1.3</td>
<td>0.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BVMT total</td>
<td>−1.31 ± 1.4</td>
<td>−1.36 ± 1.4</td>
<td>0.22</td>
<td>0.026</td>
</tr>
<tr>
<td>BVMT delay</td>
<td>−1.33 ± 1.7</td>
<td>−1.43 ± 1.7</td>
<td>0.35</td>
<td>0.007</td>
</tr>
<tr>
<td>PASAT</td>
<td>−0.59 ± 1.2</td>
<td>−0.32 ± 1.2</td>
<td>0.38</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SDMT</td>
<td>−1.58 ± 1.6</td>
<td>−1.42 ± 1.4</td>
<td>0.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DKEFS sorts</td>
<td>−0.86 ± 1.2</td>
<td>−0.66 ± 0.92</td>
<td>0.29</td>
<td>0.005</td>
</tr>
<tr>
<td>DKEFS Description</td>
<td>−0.90 ± 1.3</td>
<td>−0.66 ± 0.99</td>
<td>0.25</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Data for all tests are shown as Z-scores (mean ± SD). Partial r and P-values are for partial correlations between GMV and the NP variable correcting for age. This analysis does not include rs6265 genotype as a variable.

Figure 2. The histograms of the PASAT Z-score values for the Met66 allele (upper panel) and Val66Val groups (lower panel). The curves in each figure are the normal distributions corresponding to the histograms. The dashed line is the peak position of the normal distribution.

Met66 allele (12), which may explain the sensitivity of the PASAT to the Met66 allele in our study.

Recently, Liguori et al. (31) showed in a group (n = 50) of Italian RRMS patients that the Met66 allele was associated with a lower GM volume (P = 0.005). However, no BDNF-related impact on global neuropsychological functions was found in either RRMS patients or controls (31). The differences between our results and those in the Liguori et al. (31) study may be the result of: (i) the limited sample size of the Liguori et al. study and (ii) our patients were on disease modifying therapies for 5.3 ± 3.4 years, whereas the cohort in the Liguori et al. study was not treated.

Evidence for the beneficial effects of the available disease-modifying therapies for MS on preserving GMV (32) is now emerging and a role for neurotrophic factors in these beneficial treatment effects is suggested by experimental studies. We conducted additional linear regression analysis that included treatment groups—IFN, glatiramer acetate (GA) and chemotherapy—and obtained results similar to those reported. Nerve growth factor (33,34) and BDNF (35) are potential candidates for mediating IFN-β benefits on GM atrophy, whereas GA may confer beneficial effects by increasing BDNF production. Recent studies have reported that reduced levels of BDNF in the serum and cerebrospinal fluid of RRMS patients were reversed by therapy with GA (36,37).

A protective role for the Met66 allele, similar to those in our study, was found in a group of SLE patients without CNS involvement (n = 59) evaluated with an NP battery that included memory, attention/executive function, visuospatial skills, motor function and psychomotor speed tests (38). Patients carrying the Met66 allele showed a better cognitive performance in the psychomotor speed and motor domains (38). Interestingly, all the SLE patients in the SLE study were on prednisone therapy and the protective effect of the BDNF Met66 allele was maintained after controlling for the prednisone dosage. The PASAT, where our Met66 allele effects were mostly seen, does not involve motor function but it is heavily reliant on processing speed. In its autoimmune nature and the occurrence of neurocognitive impairments related to multifactorial CNS damage, SLE bears resemblance to MS (39).

It is interesting that the anatomical and functional effects of the Val66Met BDNF polymorphism in healthy individuals are most apparent in hippocampal formation and in prefrontal cortex, two brain regions that show abundant expression of BDNF and that are central to lifelong neuroplastic adaptations related to learning and memory (6,10,26). The impact of BDNF in MS could potentially be very different from that in healthy individuals because the pathophysiological milieu of the MS lesion contains immune cells and the disease process has deleterious effects on neurons and non-neuronal cells such as microglia, astrocytes and oligodendrocytes. As noted in Introduction, BDNF is found to be expressed in the infiltrating T-cells, macrophages as well as in neurons and activated astrocytes in active MS lesions (19–21). The TrkB BDNF receptor is expressed on neurons around the edges of active lesions (19), whereas oligodendrocytes and oligodendrocyte precursors, which are critical cells in myelination, express and even up-regulate the p75 neurotrophin receptor (NTR)
BDNF genotyping

DNA was obtained from peripheral blood mononuclear cells preserved in TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) using manufacturer’s instructions (56).

The valine/methionine SNP in BDNF was characterized using the Assays-on-Demand genotyping kit (Applied Biosystems, Redwood City, CA, USA). The fluorescent TaqMan oligonucleotide probes in the Assays-on-Demand genotyping kit are labeled with either VIC or FAM and specifically discriminate between the G and the A variants of BDNF rs6265. Genotying was performed according to manufacturer’s instructions on an MX4000 (Stratagene) real-time thermal cycler and the fluorescence outputs were analyzed using the MX4000 software. Non-template controls produced negligible background signals and excellent amplification and accurate genotyping calls were obtained using this method.

We also PCR-amplified DNA fragments (forward primer: 5'-AAA CAT CCG AGG ACA AGG TG; reverse primer: 5'-AGA AGA GGA GGC TCC AAA GG) spanning the Val66Met polymorphism using from nine samples: three each of wild-type (G/G or Val66Val), heterozygous (G/A or Val66Met) and homoyzgous (A/A or Met66Met), based on the allele discrimination assays. The PCR products were confirmed using agarose gel electrophoresis and sequenced in both directions. The electropherograms from the sequencer were analyzed using the PolyPhred program (57) and the samples were called as wild-type, heterozygous or homozygous. The agreement between the sequencing results and allele discrimination was 100% and we used the allele discrimination for remaining samples. In addition to non-template control, three sequenced reference samples—one each from G/G, G/A and A/A individuals—were included in all allele discrimination runs.

MRI acquisition and analysis

Quantitative MRI analysis was available for all 209 patients analyzed for the BDNF Val66Met polymorphism. Patients underwent brain MRI using a 1.5 T General Electric Signa 4 × /L× scanner. T2-weighted image (WI), diffusion-weighted imaging (DWI), 3D-spoiled-gradien recalled T1-WI, spin-echo T1-WI with and without gadolinium (Gd) contrast, fast, attenuated inversion recovery, proton density (PD) and PD with magnetization transfer (MT) pulse images were obtained. The details of the MRI acquisition protocol are in Supplementary Material.

Image analysis was performed in the Buffalo Neuroimaging Analysis Center (BNAC), Department of Neurology, University at Buffalo, Buffalo, NY, USA. The image analysis was blinded to patients’ clinical characteristics and the BDNF genotype. At all stages of automated analyses, quality-control montage imaging output analyses files were examined by an expert

...
observer to verify good performance of the automated algorithms.

The number of brain T1 Gd-positive lesions was based on manual tracing on the digital films (58). The T2-, T1- and Gd LVs were measured using a semi-automated edge detection contouring-thresholding technique that was manually corrected for the region of interest, as previously described (59).

For brain extraction and tissue segmentation, we utilized the SIENAX cross-sectional brain atrophy analysis tool (60,61). Compartment-specific absolute volumes were then quantified and the NBV, NGMV and NWMV were obtained, as reported previously (62).

The details of the DWI method have been described elsewhere (62,63). The MPD values were calculated.

The MT post-processing was completely automated (64). The detailed description of the MTR analysis methods is provided in Supplementary Material. The MTR of T2 and T1-LVs, whole brain MTR, NABT MTR, NAWM MTR and NAGM MTR were obtained. Given the superior reliability of mean MTR, and in order to minimize the number of multiple statistical tests, the analyses emphasized the mean MTR measure.

**Neuropsychological testing protocol**

Neuropsychological testing was conducted in accordance with consensus standards for evaluation of MS patients (65). It is well established that defects in processing speed, working memory, new learning and executive control are most common in MS, whereas basic abilities in the domains of language and perception are more often preserved (62,66). For this reason, our analysis of cognitive capacity was restricted to tests measuring the aforementioned domains.

Processing speed and working memory were evaluated using modified versions of the Symbol Digit Modalities Test (SDMT) (67) and the PASAT (68). The SDMT is a test that emphasizes the speed of information processing in the visual modality. The SDMT presents a series of nine symbols, each of which is paired with a single digit in a key at the top of the number associated with each unpaired symbol as fast as possible. The total number of correct responses in 90 s is recorded as the primary dependent measure. The SDMT is not only sensitive to MS-associated cognitive disorder, it has also been exceptionally correlated with brain imaging (69,70). The PASAT also measures processing speed and divided attention (68) but in the auditory modality. The PASAT requires patients to monitor a series of 61 audiotaped digits while adding each consecutive digit to the one immediately preceding it (71). It is widely recognized as being sensitive to cognitive changes in MS patients and the 3 s version is a component of the MS functional composite (MSFC), a clinical outcome measure composed of quantitative measures of leg, arm/hand and cognitive function (72) (Table 4).

Learning and memory were assessed with the California Verbal Learning Test, second edition (CVLT-II) (73) and the Brief Visuospatial Memory Test-Revised (BVMT-R) (74). The CVLT-II is an auditory/verbal learning and memory test. Its reliability and validity are well established and it is sensitive to impairment in a number of diseases affecting the CNS including MS (75,76). Participants are asked to learn as many of 16 words as possible over five learning trials. Delayed recall is assessed following a 20–25 min delay. The BVMT-R is a measure of visual/spatial learning and memory. The BVMT-R is sensitive to cerebral disease in general (74) and has proven to be sensitive to MS-associated impairment in recent research (77). Like the CVLT-II, this test includes a total learning score and a delay recall score.

The Delis–Kaplan Executive Function System (DKEFS) Sorting Test is a measure of higher executive function (78). This task requires the sorting of cards into as many categories as possible in 4 min. We recorded the patient’s ability to sort the cards (i.e. number of correct sorts) and their capacity to describe verbally the concepts behind the sorts (i.e. description score). This test has established validity in MS (79).

Premorbid IQ was estimated using the North American Adult Reading Test (NAART) (80).

**Data analysis**

The SPSS (SPSS Inc., Chicago, IL, USA) statistical program was used. The cube root transformation was applied to T2-LV and T1-LV prior to statistical analysis (81). The linear regression analysis with gender, presence/absence of progressive MS (secondary progressive and primary progressive MS were categorized as progressive MS), age, disease duration and treatment duration variables was used for analyzing the dependence of MRI parameters and EDSS on BDNF Val66Met genotype. The linear regression analysis for analyzing the dependence of NP parameters on BDNF Val66Met genotype used the same variables but included in addition, the number of years of education.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

**ACKNOWLEDGEMENTS**

The first and corresponding authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The research was supported from grant RG3743 from the National Multiple Sclerosis Society.

**Conflict of Interest statement.** Dr R.Z. received personal compensation from Teva Neuroscience, Biogen Idec Asperva, Pfizer and Serono for speaking and consultant fees. These were unrelated to the research in this manuscript. Dr R.Z. received financial support for research activities from National Institute of Health, National Multiple Sclerosis Society, National Science Foundation, Biogen Idec Teva Neuroscience, Asperva and Jog for the Jake Foundation. Dr B.W.-G. received honoraria and compensation from Teva Neuroscience, Biogen Idec Berlex/Bayer and Serono for speaking and consultant fees. These were unrelated to the research in this manuscript. Dr B.W.-G. received financial support for research activities from National Institutes of Health, National Multiple Sclerosis Society.
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