

ARTICLE



Effect of BDNF Val66Met polymorphism on hippocampal subfields in multiple sclerosis patients

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Brain-derived neurotrophic factor (BDNF) Val66Met polymorphism was shown to strongly affect BDNF function, but its role in modulating gray matter damage in multiple sclerosis (MS) patients is still not clear. Given BDNF relevance on the hippocampus, we aimed to explore BDNF Val66Met polymorphism effect on hippocampal subfield volumes and its role in cognitive functioning in MS patients. Using a 3T scanner, we obtained dual-echo and 3DT1-weighted sequences from 50 MS patients and 15 healthy controls (HC) consecutively enrolled. MS patients also underwent genotype analysis of BDNF, neurological and neuropsychological evaluation. Hippocampal subfields were segmented by using Freesurfer. The BDNF Val66Met polymorphism was found in 22 MS patients (44%). Compared to HC, MS patients had lower volume in: bilateral hippocampus-amygdala transition area (HATA); cornu ammonis (CA)1, granule cell layer of dentate gyrus (GCL-DG), CA4 and CA3 of the left hippocampal head; molecular layer (ML) of the left hippocampal body; presubiculum of right hippocampal body and right fimbria. Compared to BDNF Val66Val, Val66Met MS patients had higher volume in bilateral hippocampal tail; CA1, ML, CA3, CA4, and GCL-DG of left hippocampal head; CA1, ML, and CA3 of the left hippocampal body; left HATA and presubiculum of the right hippocampal head. In MS patients, higher lesion burden was associated with lower volume of presubiculum of right hippocampal body; lower volume of left hippocampal tail was associated with worse visuospatial memory performance; lower volume of left hippocampal head with worse performance in semantic fluency. Our findings suggest the BDNF Val66Met polymorphism may have a protective role in MS patients against both hippocampal atrophy and cognitive impairment. BDNF genotype might be a potential biomarker for predicting cognitive prognosis, and an interesting target to study for neuroprotective strategies.

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INTRODUCTION

During the last decade, brain plasticity was intensively studied for its critical role in overcoming brain damage, explaining the discrepancy between clinical and neuroradiological features in multiple sclerosis (MS) patients [1, 2]. However, there are strong interindividual differences among MS patients in their capacity to compensate for brain damage, which points to the role of genetic factors.

The brain-derived neurotrophic factor (BDNF) is known as the most relevant neurotrophic factor involved in brain plasticity. BDNF is secreted from dendrites to axons and from axons to dendrites, in autocrine loops, and across long distances through neural circuits [3, 4]. It may play a role in the refinement of active neural pathways through activity-dependent strengthening of co-active synapse terminals and elimination of inactive terminals [5]. The human BDNF gene has only one frequent, non-conservative polymorphism (dbSNP number rs6265): a single nucleotide polymorphism (SNP) at nucleotide 196 (G/A) resulting in an

amino acid substitution (valine to methionine) at codon 66 (hence named Val66Met). The Val66Met polymorphism was shown to interfere with the intracellular trafficking and the activity-dependent secretion of BDNF [6, 7].

The anatomical effect of Val66Met polymorphism on healthy individuals is most apparent in hippocampal formation and prefrontal cortex. These brain regions are involved in learning and memory, which require neuroplasticity, hence they show abundant expression of BDNF [6, 8, 9]. Moreover, the Val66Met polymorphism was associated with reduced volume of these structures in healthy controls [10].

To the opposite, previous studies on MS patients have shown contrasting effects of the BDNF Val66Met polymorphism on grey matter (GM) volume, also including protective effects [11–14]. Indeed, while earlier studies [14] suggested Val66Met polymorphism might be a risk factor for GM atrophy, more recent studies demonstrated a protective role against GM damage [12, 13, 15]. This peculiar effect of BDNF Val66Met polymorphism is probably

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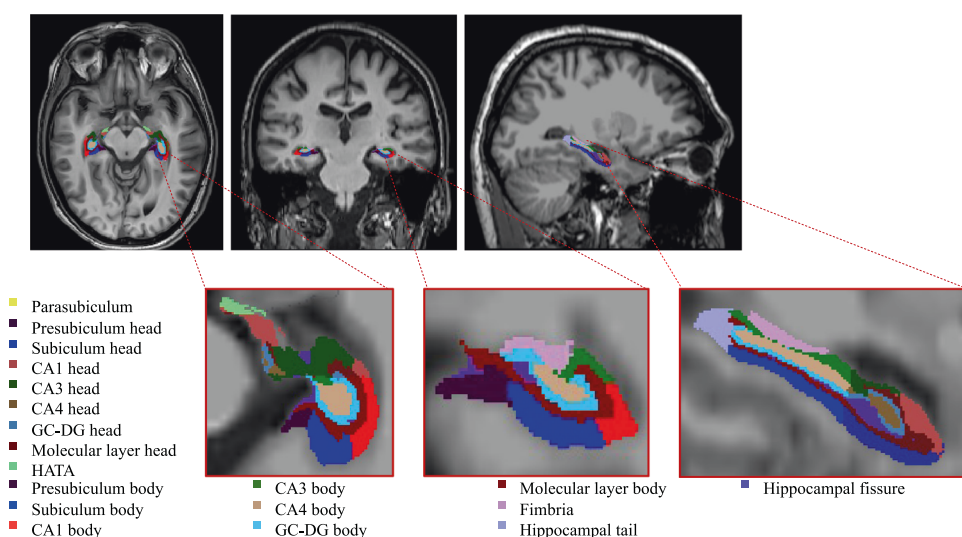


Fig. 1 Graphical representation of hippocampal subfields segmentation. Axial, coronal, and sagittal views of hippocampal subfields obtained in a healthy subject enrolled in the study. Hippocampal subfields are overlaid on the corresponding T1-weighted image by using FreeView visualization tool (<https://surfer.nmr.mgh.harvard.edu/fswiki/FreeviewGuide/>). CA cornus ammonis, GC-DG granule cell layer of dentate gyrus, HATA hippocampus-amygdala transitional area.

related to the secretion of BDNF by immune cells and to interactions of its polymorphism with the dysregulated immune system of patients with MS.

Within the hippocampal formation, regions with different vulnerability to MS-related damage have been individuated [16], as well as regions prominently involved in neurogenesis, synaptic plasticity, and long-term potentiation (LTP) likely to be more susceptible to BDNF effects.

Against this background, we aimed to assess the effect of the BDNF Val66Met polymorphism on the volume of hippocampal subfields in MS patients, and to investigate the potential association of hippocampal changes with cognitive functioning.

METHODS

Subjects

We recruited 50 consecutive right-handed adult MS patients (42 relapsing-remitting and eight progressive) [17, 18] followed at Careggi University Hospital, Florence, division of Neurology. Exclusion criteria were: history of other neurological/medical disorders in addition to MS, use of antidepressants or other psychoactive drugs, history of learning disability, severe head trauma, alcohol or drug abuse, and relapse or corticosteroid use within four weeks preceding neuropsychological and MRI assessment.

Fifteen right-handed healthy subjects (HC) with no previous history of neurological dysfunction and a normal neurological examination were also consecutively enrolled as a control group.

Clinical assessment

Neurological evaluations—including ongoing treatments, relapses, and disability level assessed every three months on the Expanded Disability Status Scale (EDSS) [19]—were collected from the disease onset to the time of study enrollment. In addition, a complete neurological evaluation with EDSS score rating was performed at the time of MRI. In order to assess disease progression, the progression index (PI) was calculated as the difference between the last EDSS and the baseline EDSS scores, divided by the disease duration.

Neuropsychological evaluation

A complete neuropsychological evaluation [20] was performed in MS patients through the administration of Rao's Brief Repeatable Battery (BRB) [20] and the Stroop Color Word Test (SCWT) [21]. The BRB assesses the most frequently impaired cognitive domains in MS [20], incorporating tests of verbal learning and memory [Selective Reminding Test (SRT) including

Long-Term Storage (SRT-LTS), Consistent Long-Term Retrieval (SRT-CLTR) and delayed recall (SRT-D)]; visual/spatial learning and memory [10/36 Spatial Recall Test (SPART) and its delayed recall (SPART-D)]; complex attention and information processing speed [Paced Auditory Serial Addition Test (PASAT) and Symbol Digit Modalities Test (SDMT)]; and verbal fluency on semantic stimulus [Word List Generation (WLG)]. The SCWT [21] assesses complex attention and aspects of executive functioning such as the ability to inhibit cognitive interference, thus integrating [20] the BRB. Corrected scores for age, sex, and education according to normative values [20] were calculated for each test. Finally, based on a population of age- and gender-matched HC ($n = 90$, females = 69, mean age [standard deviation] = 41.6 [10.4] years), z-scores for each cognitive test were calculated. A threshold of mean z-score < -1.5 was used to distinguish "severely" and "mildly" reduced performance at each test [22].

The Fatigue Severity Scale (FSS) [23] and the Montgomery and Asberg Depression Scale (MADRS) [24] were also administered during the evaluation, in order to assess fatigue and depression.

Genetic analysis: genotyping of BDNF

Blood samples were obtained from MS patients at the time of study enrollment. As previously described [25], the presence of the BDNF Val66Met polymorphism was determined by polymerase chain reaction (PCR), amplifying the DNA obtained from patients' leukocytes. Subjects' DNA was isolated from peripheral blood using the automatic standardized method (QIAcube, QIAGEN). The G→A nucleotide substitution, identifying the Val→Met amino acid change, was assayed by the High Resolution Melting Analysis (HRMA). PCR primers were 5'-ACTCTGGAGAGCGT GAATGG-3' and 5'-ACTACTGAGCATCACCTGGA-3'. Genotypes were identified by sequencing (310 ABI PRISM Genetic Analyzer, Applied Biosystem).

MRI acquisition

A 3.0 Tesla Philips Intera MR scanner with dedicated head coil (Philips Medical System, Best, The Netherlands) was used for MRI acquisition. The following MRI sequences of the brain were acquired from all subjects during a single session: (a) 3DT1-weighted turbo field echo (repetition/echo time = 25/4.6 ms; echo train length = 1; flip angle = 30°; matrix size = 256 × 256; field-of-view = 230 × 230 mm²; 220 contiguous, axial slices with voxel size = 1 × 1 × 1 mm); (b) dual-echo turbo spin-echo yielding proton density and T2-weighted images (repetition/echo time = 2599/16.80 ms, echo train length = 6; flip angle = 90°, matrix size = 256 × 256, field-of-view = 240 × 240 mm², 44 axial 3 mm-thick slices). For all sequences, the slices were positioned to run parallel to a line that joined the most infero-anterior and infero-posterior margins of the corpus callosum. MRI scans were visually assessed and repeated in case of artifacts.

Table 1. Main clinical, demographic and conventional MRI features of healthy controls and multiple sclerosis patients.

	Healthy controls	MS patients	<i>p</i> values	BDNF Val66Val MS patients	BDNF Val66Met MS patients	<i>p</i> values
Number of participants	15	50	–	22	28	–
Mean age (SD) [years]	40.6 (9.9)	41.5 (10.8)	0.83	41.8 (10.6)	41.0 (9.4)	0.89
Female/Male	8/7	40/8	0.02	26/2	15/7	0.02
Median EDSS (IQR)	–	1.5 (1.0–2.5)	–	1.0 (1.0–2.5)	1.5 (1.0–3.0)	0.15
Phenotype (RRMS/PMS)	–	42/8	–	25/3	17/5	0.46
Mean PI (SD)	–	0.04 (0.12)	–	0.04 (0.13)	0.03 (0.11)	0.63
Median disease duration (IQR) [years]	–	10.2 (4.8–19.3)	–	9.1 (4.7–16.4)	15.4 (5.2–21.2)	0.33
Ongoing DMT (none/moderate/high efficacy) ^a	–	4/31/15	–	2/17/9	2/14/6	0.91
Median T2 LV (IQR) [mL]	–	2.2 (1.3–6.7)	–	1.8 (0.9–6.7)	2.4 (1.9–5.8)	0.43
Mean NBV (SD) [mL]	1456 (70)	1415 (71)	0.04	1428 (65)	1398 (76)	0.23
Mean NGMV (SD) [mL]	760 (48)	741 (45)	0.21	752 (40)	725 (48)	0.22
Mean NWMV (SD) [mL]	696 (30)	673 (35)	0.03	675.0 (33)	672.4 (38)	0.58
Mean normalized left hippocampal volume (SD) [mL]	2.5 (0.3)	2.4 (0.2)	0.08	2.4 (0.2)	2.5 (0.2)	0.01
Mean normalized right hippocampal (SD) [mL]	2.5 (0.3)	2.4 (0.2)	0.09	2.4 (0.2)	2.5 (0.2)	0.03

MS multiple sclerosis, SD standard deviation, EDSS Expanded Disability Status Scale, PI Progression Index, IQR interquartile range, LV lesion volumes, NBV normalized brain volume, NGMV normalized gray matter volume, NWMV normalized white matter volume.

^amoderate efficacy: any interferon-beta preparation, glatiramer acetate, teriflunomide, dymethylfumarte; high efficacy: fingolimod, natalizumab, ocrelizumab.

MRI analysis

T2-hyperintense lesion volumes were measured on proton density images, using a semi-automated local thresholding segmentation technique (Jim 8, Xinapse Systems, Colchester, United Kingdom). Normalized brain (NBV), WM (NWMV) and GM (NGMV) volumes were measured on lesion-filled [26] 3DT1-weighted images using SIENAX software.

Hippocampal segmentation

The hippocampal module [27] of the FreeSurfer software, version 7.1.1 (<http://surfer.nmr.mgh.harvard.edu>), was applied to lesion-filled 3DT1-weighted images according to the cross-sectional [28] standard pipeline (Fig. 1). Global hippocampal volume and volumes of hippocampal subfields were estimated and normalized for total intracranial volume. We followed the proposed quality control procedure guidelines for the FreeSurfer-based segmentation of the hippocampal subregions designed for the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) consortium [29].

Statistical analysis

Between-group comparisons of demographic, clinical, neuropsychological and conventional MRI variables were performed with Fischer exact test for categorical variables, and Mann–Whitney, *t* test or age-, sex-, disease duration- and phenotype-adjusted (for MS patients) linear models for continuous variables, as appropriate according to normality distribution assessed by visual inspection and Shapiro–Wilk test.

Volumes of the hippocampi and their subfields were compared between MS patients and HC, between MS patients grouped by BDNF polymorphism and HC, and between MS patients with and without BDNF Val66Met polymorphism by using age-, sex-, disease duration- and phenotype-adjusted (for MS patients) linear models.

Age-, sex-, disease duration- and phenotype-adjusted linear models were also used to explore the relationship of hippocampal subfield volumes showing significant abnormalities in MS patients vs HC with clinical, conventional MRI, and neuropsychological features. Multivariate regression models, including EDSS, NBV, NWMV, and NGMV in addition to hippocampal subfield volumes and the above-specified covariates, were adopted to assess the role of hippocampal subfield volumes as independent predictors of cognitive performance. A stepwise variable selection procedure was used ($p = 0.15$ for entry and $p = 0.05$ to remain in the model). Finally, we performed a

regression analysis exploring the role of the BDNF polymorphism on neuropsychological variables, while assessing the role of hippocampal subfield volumes as mediators.

False-discovery-rate (Benjamini–Hochberg procedure) correction was separately applied to account for the overall number of each group of pairwise comparisons and pairwise contrasts, in between-group comparisons and regression analysis, respectively. For all analyses, the statistical threshold was set at p value <0.05 . Statistical analysis was performed with the R software, version 4.0.2.

RESULTS

Clinical and conventional MRI measures

Table 1 summarizes the main demographic, clinical and conventional MRI features of the study cohort. Compared to HC, MS patients were well matched in terms of age distribution, although they had a preponderance of female subjects ($p = 0.02$). They had brain ($p = 0.04$) and white matter ($p = 0.03$) atrophy, but no significant NGMV reduction. Compared to HC, MS patients showed a trend towards left ($p = 0.08$) and right ($p = 0.09$) normalized hippocampal volume reduction. Twenty-two (44%) MS patients were carriers of the BDNF Val66Met polymorphism. Compared to Val66Val, Val66Met MS patients included a greater proportion of male subjects ($p = 0.02$), while no significant differences were observed in terms of age, disability, disease duration, phenotype, and disease-modifying treatment. Compared to HC, Val66Val MS patients showed lower left ($p = 0.02$) and right ($p = 0.05$) normalized hippocampal volume, but no significant differences in NBV, NGMV, NWMV. Compared to HC, Val66Met patients showed no significant difference in normalized hippocampal volume as well as in NBV, NGMV, NWMV. Compared to Val66Val, Val66Met MS patients showed higher left ($p = 0.01$) and right ($p = 0.05$) normalized hippocampal volume, but no significant differences in NBV, NGMV, NWMV.

Neuropsychological measures

Table 2 summarizes the main neuropsychological features of MS patients. MS patients showed mildly reduced cognitive

Table 2. Neuropsychological variables, reported as z-scores, in the whole group of multiple sclerosis (MS) patients and in MS patients grouped according to the presence of brain-derived neurotrophic factor (BDNF) Val66Met polymorphism.

	All MS patients	BDNF Val66Val MS patients	BDNF Val66Met MS patients	p value ^a
Education [years]	13.0 (2.8)	13.8 (2.8)	12.1 (2.5)	0.14
FSS	4.0 (2.1)	3.8 (2.1)	4.2 (2.3)	0.75
MADRS	5.3 (3.7)	4.9 (2.2)	5.8 (5.1)	0.54
Verbal learning				
SRT-LTS	−0.57 (0.84)	−0.69 (0.87)	−0.41 (0.82)	0.56
SRT-CLTR	−0.56 (0.79)	−0.46 (0.76)	−0.69 (0.83)	0.76
SRT-D	−0.19 (0.97)	−0.27 (1.03)	−0.09 (0.06)	0.74
Visuospatial learning				
SPART	−0.46 (1.09)	−0.68 (1.09)	−0.17 (1.05)	0.26
SPART-D	−1.02 (0.96)	−1.52 (0.95)	−0.55 (0.78)	0.03
Attention and information processing speed				
SDMT	−0.27 (1.30)	−0.36 (1.53)	−0.16 (0.98)	0.91
PASAT3	−0.70 (1.37)	−0.77 (1.31)	−0.62 (1.48)	0.76
PASAT2	−0.82 (1.83)	−1.21 (2.08)	−0.30 (1.37)	0.13
Language				
WLG	−0.39 (0.76)	−0.35 (0.71)	−0.41 (0.85)	0.99
Executive functions				
SCWT	−0.33 (1.25)	−0.51 (0.82)	−0.12 (1.63)	0.42

MS multiple sclerosis, BDNF brain-derived neurotrophic factor, FSS Fatigue Severity Scale, MADRS Montgomery-Asberg Depression Scale, SRT Selective Reminding Test, LTS Long-Term Storage, CLTR Consistent Long-Term Retrieval, D delayed recall, SPART Spatial Recall Test, PASAT Paced Auditory Serial Addition Test, SDMT Symbol Digit Modalities Test, WLG Word List Generation, SCWT Stroop Color Word Test.

^aadjusted for disease duration and phenotype and false-discovery-rate corrected.

performance in all cognitive tests, compared to HC. No difference in education was observed between Val66Val and Val66Met MS patients. Only in SPART-D, BDNF Val66Val MS patients showed severely reduced performance compared to HC, and Val66Met MS patients showed better performance compared to Val66Val MS patients ($p = 0.02$). No significant differences were observed between Val66Val and Val66Met MS patients in the remaining cognitive tests, as well as in fatigue and depression scores.

Hippocampal subfields

Compared to HC, MS patients had lower volumes in: left ($p = 0.04$) and right ($p = 0.04$) hippocampus-amygdala transition area (HATA); cornu ammonis (CA)1 ($p = 0.04$), granule cell layer of dentate gyrus (GCL-DG) ($p = 0.04$), CA4 ($p = 0.05$) and CA3 ($p = 0.05$) of left hippocampal head; molecular layer (ML) ($p = 0.05$) of left hippocampal body; presubiculum ($p = 0.05$) of right hippocampal body and right fimbria ($p = 0.03$). Compared to HC, BDNF Val66Val MS patients had lower volume in: left ($p = 0.01$) and right ($p = 0.01$) HATA; left ($p = 0.05$) and right ($p = 0.05$) hippocampal tail; CA1 ($p = 0.04$), GCL-DG ($p = 0.04$), CA4 ($p = 0.05$), CA3 ($p = 0.01$) of left hippocampal head; ML ($p = 0.05$) of left hippocampal body; CA3 ($p = 0.05$) of right hippocampal head, presubiculum ($p = 0.02$) of right hippocampal body, and right fimbria ($p = 0.05$). Compared to HC, BDNF Val66Met MS patients had lower volume in: left ($p = 0.02$) and right ($p = 0.01$) HATA; left CA3 ($p = 0.04$) of hippocampal head; and presubiculum of right hippocampal body ($p = 0.01$).

Compared to BDNF Val66Val, Val66Met MS patients had higher volume in: left ($p = 0.03$) and right ($p = 0.01$) hippocampal tail; CA1 ($p = 0.01$), ML ($p = 0.01$), CA3 ($p = 0.02$), CA4 ($p = 0.01$) and GCL-DG ($p = 0.02$) of left hippocampal head; CA1 ($p = 0.02$), ML ($p = 0.01$) and CA3 ($p = 0.01$) of left hippocampal body, left HATA ($p = 0.01$) and presubiculum of right hippocampal head ($p = 0.05$) (Table 3).

Correlation analysis

No significant associations were found between hippocampal subfield abnormalities with clinical and conventional MRI variables, except for T2 lesion volume, which was associated with lower volume of presubiculum of right hippocampal body ($\beta = -3.76$; $p = 0.05$). Table 4 summarizes significant associations between hippocampal subfield volumes and neuropsychological variables. Significant associations were found between:

Worse SPART-D performance with lower left hippocampal tail volume ($\beta = 0.39$; $p = 0.04$);

Worse WLG performance with lower volume of GCL-DG ($\beta = 0.39$; $p = 0.04$), CA3 ($\beta = 0.40$; $p = 0.04$) and CA4 ($\beta = 0.43$; $p = 0.02$) belonging to left hippocampal head.

Multivariate analysis, including NBV, NWMV and NGMV in addition to hippocampal subfield volumes and clinical variables, confirmed the role of left hippocampal tail as an independent predictor of SPART-D performance ($\beta = 0.44$, 95% CI = 0.45–0.47; $p = 0.05$) and CA4 of left hippocampal head as an independent predictor of WLG performance ($\beta = 0.62$; 95% CI = 0.59–0.65; $p = 0.006$).

Left hippocampal tail volume resulted as mediator of BDNF polymorphism effect on SPART-D performance: introducing left hippocampal tail volume as covariate, BDNF polymorphism had no significant effect on SPART-D performance ($p = 0.10$).

DISCUSSION

In this study, we explored the impact of the BDNF Val66Met polymorphism on hippocampal formation in MS patients, and its clinical and neuropsychological correlates. The analysis was separately performed for each hippocampal subfield, considering both their different susceptibility to MS-related damage [16, 30, 31] and their functional specialization. These features,

Table 3. Mean and standard deviations of left and right hippocampal subfield volumes are reported for multiple sclerosis (MS) patients and healthy controls as well as for MS patients grouped according to the presence of BDNF Val66Met polymorphism.

Left Hippocampus	HC	MS patients	p value vs HC	BDNF Val66Val MS patients	p value vs HC	BDNF Val66Met MS patients	p value vs HC	Val66Met vs Val66Val p value
Hippocampal tail [mL]	0.41 (0.07)	0.39 (0.05)	0.73	0.38 (0.04)	0.05	0.40 (0.05)	0.75	0.03
Subiculum body [mL]	0.17 (0.02)	0.17 (0.02)	0.96	0.17 (0.02)	0.82	0.16 (0.02)	0.88	0.35
CA1 body [mL]	0.10 (0.01)	0.09 (0.01)	0.24	0.09 (0.01)	0.12	0.10 (0.01)	0.46	0.02
Subiculum head [mL]	0.12 (0.02)	0.13 (0.01)	0.90	0.13 (0.01)	0.85	0.13 (0.01)	0.69	0.48
Presubiculum head [mL]	0.09 (0.01)	0.09 (0.01)	0.54	0.09 (0.01)	0.26	0.09 (0.01)	0.43	0.15
CA1 head [mL]	0.39 (0.06)	0.37 (0.04)	0.04	0.36 (0.04)	0.04	0.38 (0.04)	0.18	0.01
Presubiculum body [mL]	0.11 (0.02)	0.10 (0.02)	0.11	0.10 (0.02)	0.26	0.10 (0.01)	0.09	0.62
Parasubiculum [mL]	0.04 (0.01)	0.04 (0.01)	0.97	0.04 (0.01)	0.89	0.05 (0.01)	0.96	0.34
Molecular layer head [mL]	0.24 (0.04)	0.23 (0.02)	0.28	0.23 (0.02)	0.10	0.24 (0.02)	0.29	0.01
Molecular layer body [mL]	0.17 (0.02)	0.16 (0.02)	0.05	0.16 (0.02)	0.05	0.17 (0.02)	0.27	0.01
Granule cell layer of DG head [mL]	0.12 (0.02)	0.11 (0.01)	0.04	0.11 (0.01)	0.04	0.12 (0.02)	0.13	0.02
CA3 body [mL]	0.07 (0.01)	0.07 (0.01)	0.70	0.07 (0.01)	0.20	0.08 (0.01)	0.76	0.01
Granule cell layer of DG body [mL]	0.10 (0.01)	0.10 (0.01)	0.72	0.10 (0.01)	0.38	0.10 (0.01)	0.91	0.15
CA4 head [mL]	0.10 (0.01)	0.09 (0.01)	0.05	0.09 (0.01)	0.05	0.10 (0.01)	0.23	0.01
CA4 body [mL]	0.09 (0.01)	0.09 (0.01)	0.95	0.09 (0.01)	0.52	0.09 (0.01)	0.68	0.13
Fimbria [mL]	0.05 (0.01)	0.05 (0.01)	0.43	0.05 (0.01)	0.14	0.05 (0.01)	0.15	0.34
CA3 head [mL]	0.10 (0.02)	0.09 (0.01)	0.05	0.09 (0.01)	0.01	0.10 (0.01)	0.04	0.02
HATA [mL]	0.05 (0.01)	0.04 (0.01)	0.04	0.04 (0.01)	0.01	0.05 (0.01)	0.02	0.01
Right hippocampus	HC	MS patients	p value vs HC	BDNF Val66Val MS patients	p value vs HC	BDNF Val66Met MS patients	p value vs HC	Val66Met vs Val66Val p value
Hippocampal tail [mL]	0.42 (0.07)	0.39 (0.04)	0.24	0.38 (0.04)	0.05	0.40 (0.04)	0.15	0.01
Subiculum body [mL]	0.17 (0.02)	0.17 (0.02)	0.89	0.17 (0.02)	0.54	0.17 (0.02)	0.93	0.35
CA1 body [mL]	0.10 (0.01)	0.10 (0.01)	0.87	0.10 (0.02)	0.46	0.10 (0.01)	0.81	0.54
Subiculum head [mL]	0.13 (0.02)	0.13 (0.02)	0.96	0.13 (0.02)	0.76	0.13 (0.01)	0.80	0.34
Presubiculum head [mL]	0.09 (0.01)	0.09 (0.01)	0.55	0.09 (0.01)	0.28	0.09 (0.01)	0.67	0.05
CA1 head [mL]	0.37 (0.06)	0.35 (0.03)	0.43	0.35 (0.03)	0.21	0.36 (0.03)	0.36	0.20
Presubiculum body [mL]	0.11 (0.02)	0.10 (0.02)	0.05	0.10 (0.02)	0.02	0.10 (0.02)	0.01	0.72
Parasubiculum [mL]	0.04 (0.01)	0.04 (0.01)	0.75	0.04 (0.01)	0.72	0.05 (0.01)	0.41	0.37
Molecular layer head [mL]	0.24 (0.03)	0.23 (0.02)	0.60	0.23 (0.02)	0.36	0.23 (0.02)	0.62	0.15
Molecular layer body [mL]	0.17 (0.03)	0.16 (0.02)	0.43	0.16 (0.02)	0.19	0.16 (0.02)	0.33	0.41
Granule cell layer of DG head [mL]	0.11 (0.02)	0.11 (0.01)	0.55	0.11 (0.01)	0.11	0.11 (0.01)	0.46	0.30
CA3 body [mL]	0.07 (0.01)	0.07 (0.01)	0.95	0.07 (0.01)	0.44	0.07 (0.01)	0.54	0.20
Granule cell layer of DG body [mL]	0.10 (0.02)	0.10 (0.01)	0.73	0.10 (0.01)	0.16	0.10 (0.01)	0.77	0.41
CA4 head [mL]	0.09 (0.02)	0.09 (0.01)	0.69	0.09 (0.01)	0.16	0.09 (0.01)	0.64	0.23

Table 3 continued

Left Hippocampus	HC	MS patients	p value vs HC	BDNF Val66Val MS patients	p value vs HC	BDNF Val66Met MS patients	p value vs HC	Val66Met vs Val66Val p value
CA4 body [mL]	0.09 (0.01)	0.09 (0.01)	0.96	0.09 (0.01)	0.30	0.09 (0.01)	0.86	0.41
Fimbria [mL]	0.06 (0.01)	0.05 (0.01)	0.03	0.05 (0.01)	0.05	0.05 (0.01)	0.13	0.41
CA3 head [mL]	0.09 (0.02)	0.09 (0.01)	0.47	0.09 (0.01)	0.05	0.09 (0.01)	0.33	0.41
HATA [mL]	0.05 (0.01)	0.04 (0.01)	0.04	0.04 (0.01)	0.01	0.04 (0.01)	0.01	0.94

BDNF brain-derived neurotrophic factor, HC healthy controls, MS multiple sclerosis, CA cornu ammonis, DG dentate gyrus, HATA hippocampus amygdala transitional area.

Table 4. Results of age-, sex-, phenotype- and disease duration-adjusted linear regression models for the effect of hippocampal subfields on neuropsychological variables.

	Hippocampal subfield	β	95% CI	p
SPART-D	Left hippocampal tail	0.39	0.38, 0.40	0.04
WLG	Left granule cell layer of DG	0.39	0.37, 0.41	0.04
	Left CA4 head	0.43	0.41, 0.46	0.02
	Left CA3 head	0.40	0.38, 0.42	0.04

CI confidence interval, DG dentate gyrus, CA cornu ammonis, SPART-D Spatial Recall Test delayed recall, WLG Word List Generation.

together with the evidence of plasticity occurring in this structure, make the analysis of hippocampal subfields a powerful tool to in-vivo explore disease-related changes, including neuronal loss, neurogenesis, and functional reorganization, and the impact of the BDNF Val66Met polymorphism on these processes [32].

Compared to HC, we observed reduced volumes of several left hippocampal subfields, including CA1, GCL-DG, CA4 and CA3. These results are in line with previous findings highlighting the prominent vulnerability of CA1 [30, 33] and of CA3/CA4/DG [34] in MS. In detail, CA1 is the most affected hippocampal region in a variety of neurological disorders, and especially in MS. Neurons within the CA1 region are highly susceptible to ischemia and glutamate-mediated excitotoxicity [35], implicated in MS-related CNS damage [36]. In MS, pathological studies reported a reduction of neuronal count and size [37], decreased dendritic density, and the presence of demyelinating lesions in this area [16, 37], which translate into CA1 volume loss. Despite the relevance of CA1 volume loss in MS, a recent study demonstrated that the CA3/CA4/DG subfield of the hippocampus is the first region to become atrophic in MS, from the stage of clinically isolated syndrome [38]. Moreover, CA4/DG atrophy at the stage of clinically isolated syndrome was found to predict the atrophy of CA1 one year later, thus suggesting a possible pattern of hippocampal damage spreading. According to this hypothesis a gradient of infiltrating immune cells and cytokines might diffuse progressively from CSF to DG, then to CA1, and finally to the whole medial temporal lobe [38].

Beyond previous studies, we also showed atrophy of left ML, right presubiculum, right fimbria, and bilateral HATA in MS patients compared to HC. These results may partly be due to the adoption of an advanced hippocampal subfield segmentation technique, with heightened sensitivity. The ML contains interneurons and the apical dendrites of hippocampal pyramidal cells, thus representing an input region to hippocampal subfields, which receive a great flow of information [27, 39]. The decreased volume observed in this subfield could be due to both reduced intra- and extra-hippocampal connectivity and neuron loss in subiculum and CA fields [40]. Previous studies have already described subiculum involvement in MS, defining "subiculum" as the so-called "subicular complex", which included presubiculum, subiculum, and post-subiculum. It is the cytoarchitectonic organization of presubicular cortex (comprising six-layers [41, 42] and the dense plexus formed by afferent axons) that distinguishes the presubiculum from the neighboring subiculum and post-subiculum. Given the density of afferent axons to the presubiculum, which plays prominent roles in intrahippocampal and cortico-hippocampal pathways, it is not surprising that our study demonstrated specific atrophy of the right presubiculum within the subicular complex. The association found between reduced right presubiculum volume and higher T2 lesion burden supports the hypothesis of Wallerian degeneration originating from the damage to connecting fibers by MS WM lesions. Moreover, although not assessed in the present study, intracortical and leukocortical

demyelinating lesions are frequent in the presubiculum [16, 37], and may contribute to its morphologic alterations.

The HATA is part of the hippocampus-amygdala circuitry. The CA1 projects to the amygdala, which in turn projects to the HATA [43, 44]. Thus, this region mediates the hippocampus-amygdala interactions involved in visuospatial function and object discrimination. The fimbria, a white matter structure forming part of the fornix and projecting to the amygdala [27], is also involved in such cognitive functions. Damage to these areas might be due to MS lesions along the fornix or in the hippocampus-amygdala circuitry, which were not specifically assessed in our study. Given the polysynaptic projection of the CA1 back to the HATA, we might further speculate damage to the HATA may be partially secondary to neuronal loss in the CA1.

Looking at the effect of BDNF Val66Met polymorphism onto the hippocampal formation in MS, we found higher volumes of several hippocampal subfields in Val66Met carriers, thus suggesting a protective role of this polymorphism in MS. We investigated this polymorphism given the central role of BDNF in neurogenesis, neuronal maturation, circuit formation, and activity-dependent forms of plasticity, such as long-term potentiation (LTP) of synaptic transmission [45]. In detail, the BDNF was shown to stimulate the proliferation of neural progenitor cells and to promote long-term survival of their progeny [46–48], and intrahippocampal infusion of BDNF stimulated hippocampal neurogenesis [49–51]. Nevertheless, the main function of BDNF is to enhance synaptic transmission, facilitate synaptic plasticity and promote synaptic growth [52]. The BDNF Val66Met polymorphism has been proven to result in impaired dendritic trafficking and synaptic localization of the protein and, most importantly, an 18–30% reduction in activity-dependent BDNF secretion [6, 53]. Indeed, the BDNF Val66Met polymorphism is generally linked to lower brain and hippocampal volumes and to worse cognitive performance in neurodegenerative disorders. However, the results of our and previous studies in MS contrast with these findings in neurodegenerative disorders, showing a protective role of the BDNF Val66Met polymorphism.

A first study by Liguori et al. [14] showed that the Met66 allele was associated with lower GM volume in a group of Italian RRMS patients. Conversely, in line with our findings, subsequent studies on larger cohorts of MS patients highlighted a protective effect of the BDNF Val66Met polymorphism on GM volume [12, 54]. These results suggest an interplay between BDNF functioning and the inflammatory microenvironment. As a matter of fact, BDNF is also secreted by immune cells to promote neuronal and axonal survival or repair in the context of inflammation. In RRMS patients, higher levels of BDNF are secreted by lymphocytes during relapses (2.6-fold higher) and during the subsequent recovery phase (2-fold higher), compared with a stable remission period [55]. Moreover, the inflammatory microenvironment might exert epigenetic effects on BDNF secretion. Indeed, MS patients with a more severe inflammation showed de-methylation of its gene, leading to a higher secretion of BDNF [56].

On the other hand, the increased secretion of BDNF in MS also is likely to have negative consequences. LTP induced by BDNF facilitates glutamatergic synaptic transmission [57, 58] and, hence, glutamate-excitotoxicity with loss of oligodendrocytes and neurons. Furthermore, the overproduction of BDNF might lead to faster exploitation of brain reserve (for instance, in terms of progenitor cells and synapses), thus leading to more severe brain damage over the long-term. Against this background, reduced secretion of BDNF due to the presence of the Val66Met polymorphism may have a protective function in MS. An intriguing hypothesis is that chronically elevated levels of BDNF released by immune cells reduce the signal/noise ratio of neurally-secreted BDNF in MS, thus reducing activity-dependent function of BDNF. Another hypothesis suggests that BDNF Val66Met polymorphism, especially in an inflammatory context, is likely to alter the

proportions of the proapoptotic proBDNF and the antiapoptotic mature BDNF, enhancing the levels of the latter [59].

In line with the notion that Val66Met polymorphism only affects the BDNF activity-dependent pathways [6], in our study we found higher volumes in Val66Met vs Val66Val patients in those hippocampal subfields where BDNF is most likely to exert its function (directly or indirectly), namely the GCL-DG, CA1, and CA3. In detail, compared to HC, while Val66Met patients had reduced volume in the previously mentioned subfields, Val66Val patients had reduced volume in CA3 only, supporting the hypothesis of a protective role of the BDNF Val66Met polymorphism on these subfields. Indeed, as previously specified, BDNF plays a major role in adult neurogenesis, which is typically localized in the GCL-DG. Furthermore, this region, together with CA1 and CA3, is part of the tri-synaptic loop [60, 61], the main substrate of LTP, playing a fundamental role in learning and memory information.

The hippocampal tail and the presubiculum of hippocampal body are two well-connected areas of the hippocampus (especially with the prefrontal cortex), and also substrates of LTP. Although we did not find significant differences when comparing MS vs HC in left and right hippocampal tail volume, we observed that Val66Val – but not Val66Met – MS patients had reduced volume in this region, compared to HC. Moreover, we found higher volumes in Val66Met vs Val66Val patients in such regions, further suggesting a protective role of the BDNF Val66Met polymorphism on hippocampal subfields with high synaptic plasticity.

It is interesting to underscore the differential involvement of left and right hippocampi in our study, providing in-vivo evidence of left–right asymmetry in synaptic function and MS-related damage [62]. We might speculate the higher plasticity of the left hippocampus make it more vulnerable to glutamate-mediated excitotoxicity and remodeling phenomena secondary to disease-related damage. As converse, the right hippocampus harbors more stable synapses. Thus, it might be more likely to suffer from damage due to Wallerian degeneration, caused by axonal transection from WM lesions. In line with this hypothesis, we found a broader pattern of damage involving the left hippocampus in MS patients compared to HC, especially in areas with a high synaptic density. These results are in line with existing literature reporting a preferential left-sided lateralization of atrophy in MS [63]. Instead, in our study, the right hippocampus showed a more selective involvement of the output component and areas involved in the amygdala–hippocampus interaction. In our patients, the connection to WM lesions was supported by the association of right presubiculum volume with brain T2 lesion burden.

In line with previous studies [12, 13], we found significant associations between lower hippocampal subfield volume and worse performance on specific neuropsychological tests in MS patients, thus pointing towards a protective role of the BDNF Val66Met polymorphism. We found a significant association between better visuospatial memory performance (SPART-D) and left hippocampal tail volume. Moreover, left hippocampal tail volume resulted as a mediator of BDNF polymorphism effect on visuospatial memory performance. Although it is widely known that posterior hippocampal regions are related to visuospatial navigation [64–68], visuospatial performance is generally lateralized to the right. However, recent evidence [69] reported bilateral hippocampal involvement in visuospatial memory, with complementary roles of left and right formations. In detail, the left hippocampus mediates spatiotemporal associations between the multiple elements of episodic memory [67, 70] underlying the planning of complex routes in humans [71, 72]. Moreover, fMRI studies reported left hippocampus activation when a novel sensory stimulus is spatially or temporally different from a previously encountered input [73]. As converse, right hippocampal activation was seen only in cases when there was a complete

match between novel and previously encountered inputs [73]. Therefore, the left hippocampus might be preferentially involved in updating internal representations in response to changes in sensory experience, thus showing more intense brain plasticity phenomena.

In line with the localization of semantic processing in anterior hippocampal regions, we found significant associations between subfields belonging to left hippocampal head and semantic fluency performance on the WLG. In detail, the anterior hippocampus is involved in the integration of distinct informational elements around a central conceptual node, relating a set of ideas or experiences to a common theme, as it happens in autobiographical memory [74, 75]. This anterior distribution for semantic retrieval is likely to be subserved by anterior hippocampus connections with anterior medial temporal lobes and anterior portion of lateral temporal lobes [76–78].

This study is not without limitations. The main limitation is the small numerosity of our cohort. Also, we could not obtain BDNF genotyping on HC. There were some sex-related differences between study groups, which were accounted for by specific adjustment during statistical analysis. Only a few patients had hippocampal lesions, so we could not consider hippocampal lesion load as a marker of damage. Finally, the cross-sectional study design did not allow us to assess whether the BDNF Val66Met polymorphism is actually neuroprotective, slowing down MS-related hippocampal atrophy. The alternative hypothesis is that Val66Met MS patients may start off with a greater hippocampal volume, thus masking—in a cross-sectional setting—a similar rate of hippocampal atrophy as Val66Val MS patients. We address to future longitudinal studies to resolve the matter.

In conclusion, by adopting an advanced MRI technique combined with genetic analysis, we suggest the BDNF Val66Met polymorphism might play a protective role in MS patients, limiting hippocampal damage and possibly preventing disease-related cognitive decline. This pilot study can pave the way to longitudinal studies on the effect of the BDNF Val66Met polymorphism in MS. In future, BDNF genotyping may prove to be helpful to predict patient long-term prognosis. The study of the molecular mechanisms underlying the protective effect of Val66Met polymorphism in MS might help individuate new targets for neuroprotective therapeutic strategies.

DATA AVAILABILITY

The dataset used and analyzed during the current study is available from the corresponding Author on reasonable request.

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AUTHOR CONTRIBUTIONS

Ermelinda De Meo contributed to study concept and design, drafting/revising the manuscript, data analysis; Emilio Portaccio contributed to patient recruitment and clinical assessment; Elio Prestipino contributed to patient recruitment, clinical assessment and data analysis; Benedetta Nacmias contributed to genetic analysis; Silvia Bagnoli contributed to genetic analysis; Lorenzo Razzolini contributed to patient recruitment and clinical assessment; Luisa Pastò contributed to patient recruitment and clinical assessment; Claudia Nicolai contributed to neuropsychological assessment; Benedetta Goretti contributed to neuropsychological assessment; Angelo Bellinva contributed to patient recruitment and clinical assessment; Mattia Fonderico contributed to patient recruitment and clinical assessment; Antonio Giorgio contributed to MRI data analysis; Maria Laura Stromillo contributed to MRI data analysis; Massimo Filippi contributed to study drafting/revising the manuscript; Sandro Sorbi contributed to study drafting/revising the manuscript; Nicola De Stefano contributed to study drafting/revising the manuscript; Maria Pia Amato, MD contributed to study concept and drafting/revising the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval was received from Ethics Committee on human experimentation of the University of Florence, and written informed consent was obtained from all participants prior to study enrollment.

ADDITIONAL INFORMATION

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