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ARTICLE

Association of BDNF with restricting anorexia nervosa and minimum body mass index: a family-based association study of eight European populations

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Eating disorders (ED), such as anorexia nervosa (AN) and bulimia nervosa (BN), are complex psychiatric disorders where different genetic and environmental factors are involved. Several lines of evidence support that brain-derived neurotrophic factor (BDNF) plays an essential role in eating behaviour and that alterations on this neurotrophic system participates in the susceptibility to both AN and BN. Accordingly, intraventricular administration of BDNF in rats determines food starvation and body weight loss, while *BDNF* or its specific receptor *NTRK2* knockout mice develop obesity and hyperphagia. Case–control studies also suggest a *BDNF* contribution in the aetiology of ED: we have previously reported a strong association between the Met66 variant within the *BDNF* gene, restricting AN (ANR) and minimum body mass index (minBMI) in a Spanish sample, and a positive association between the Val66Met and −270C/T *BDNF* SNPs and ED in six different European populations. To replicate these results, avoiding population stratification

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effects, we recruited 453 ED trios from eight European centres and performed a family-based association study. Both haplotype relative risk (HRR) and haplotype-based haplotype relative risk (HHRR) methods showed a positive association between the Met66 allele and ANR. Consistently, we also observed an effect of the Met66 variant on low minBMI and a preferential transmission of the −270C/Met66 haplotype to the affected ANR offspring. These results support the involvement of BDNF in eating behaviour and further suggest its participation in the genetic susceptibility to ED, mainly ANR and low minBMI.
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Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are eating disorders (ED) with a complex and multifactorial aetiology, where environmental and genetic factors are involved.[1] Different lines of evidence suggest that brain-derived neurotrophic factor (*BDNF*), which encodes for a neurotrophin with a role in synaptic plasticity and neuronal development, participates in eating behaviour and weight regulation.[2–4] Accordingly, animal models and association studies support that alterations on this neurotrophic system could be involved in the aetiopathology of ED.[5–9] We previously screened the *BDNF* gene in 95 ED patients, identified the −270C>T and Val66Met SNPs and observed a strong association of the Met66 allele, restricting AN (ANR) and minimum body mass index (minBMI;[10]). We then aimed to replicate this study in a sample from six different European centres and found that the Met66 variant was associated to all ED subtypes (ANR, binge-eating/purging AN (ANB) and BN), and that the −270C allele had an effect on BN and late age at onset of weight loss.[11] Finally, Koizumi *et al* also described a positive association between the Met66 allele within the *BDNF* gene, ANR and BNP.[12] However, no family studies have yet been carried out to detect the participation of this neurotrophic factor in ED. To address this question and avoid potential population stratification, we performed a family-based study following two different strategies: we first carried out a haplotype relative risk (HRR) and a haplotype-based haplotype relative risk (HHRR) analyses for genotypes and alleles of both the Val66Met and the −270C/T polymorphisms,[13] and then performed a transmission disequilibrium test (TDT) to study linkage in the presence of association.[14]

We obtained a total sample of 453 ED family trios independently recruited from seven European countries participating in the European Community Framework V 'Factors in Healthy Eating' project (Austria, France, Germany, Italy, Slovenia, Spain and United Kingdom). Owing to the presence of genetic and phenotypic heterogeneity, we considered the three main ED groups of ANR, ANB and BN that have been shown to differ in various biological and psychopathological features.[15–17] We also focused the study on different phenotypical markers that may reflect the severity of the illness, such as minBMI, maximum body mass index (maxBMI) and age at onset of weight loss (AO).

Results

The Val66Met and −270C/T SNPs were in linkage disequilibrium ($D' = 0.78$) and followed Hardy–Weinberg equilibrium in ED patients ($\chi^2 = 0.055$, $P = 0.81$ for the Val66Met and $\chi^2 = 2.4$, $P = 0.12$ for the −270C/T SNPs) and their parents ($\chi^2 = 2.1$, $P = 0.14$ for the Val66Met, and $\chi^2 = 0.02$ $P = 0.9$ for the −270C/T SNPs).

We first performed an HRR analysis considering the nontransmitted alleles from the parents to the probands as a 'virtual' control group. No significant differences were observed when the −270C/T SNP was considered (data not shown). However, we found a positive association of the Met66 allele and ANR in the British ($\chi^2 = 4.3$, $P = 0.037$), Spanish ($\chi^2 = 6.5$, $P = 0.012$) and French samples ($\chi^2 = 3.9$, $P = 0.035$; Table 1). Once we discarded the presence of genetic heterogeneity by comparing the nontransmitted alleles from the different centres ($\chi^2 = 9.4$, df = 6, $P = 0.15$ for the Val66Met and $\chi^2 = 3.7$, df = 6, $P = 0.72$ for the −270C/T SNPs), the combined analysis of the total sample of European trios confirmed a significant association between ANR and the Met66 allele of the Met66Val SNP ($\chi^2 = 5.1$, $P = 0.015$). These results were also observed when we compared the Val66Met allele frequencies by the HHRR test ($\chi^2 = 4.6$, $P = 0.019$, OR = 1.4; Table 1). After Bonferroni correction, taking into account two different SNPs, the Met66 allele was still positively associated to ANR.

We then analysed the ANB group and observed a positive association between this clinical subtype and the Val66 allele when both genotype ($\chi^2 = 7.3$, $P = 0.009$) and allele frequencies of the German population were considered ($\chi^2 = 8.1$, $P = 0.0048$, OR = 8.4; Table 2). However, no significant differences were found when we analysed all the ANB European trios or the BN sample.

The TDT analysis showed that only 60 and 14% of the total sample of 453 ED trios were informative for the Val66Met and the −270C>T polymorphisms, respectively.

Table 1 Genotypic and allelic distribution of the Val66Met SNP of the *BDNF* gene in patients with restricting AN and 'virtual' controls

	Restricting AN											
	n *Genotypes (%)*[a]							n *Alleles (%)*[b]				
	Probands			*Pseudocontrols*[c]			P-*value*	*Probands*		*Pseudocontrols*[c]		P-*value (OR)*
	ValVal	*ValMet*	*MetMet*	*ValVal*	*ValMet*	*MetMet*		*Val*	*Met*	*Val*	*Met*	
Italian	11 (52.4)	10 (47.6)	0	11 (52.4)	9 (42.9)	1 (4.7)	NS	32 (76.2)	10 (23.8)	31 (73.8)	11 (26.2)	NS
German	48 (75.0)	13 (20.3)	3 (4.7)	46 (71.9)	14 (21.9)	4 (6.2)	NS	109 (85.2)	19 (14.8)	106 (82.8)	22 (17.2)	NS
British	14 (53.8)	10 (38.5)	2 (7.7)	21 (80.8)	5 (19.2)	0	0.037	38 (73.0)	14 (27.0)	47 (90.4)	5 (9.6)	0.02* (3.5)
Spanish	5 (25.0)	14 (70.0)	1 (5.0)	13 (65.0)	6 (30.0)	1 (5.0)	0.012*	24 (60.0)	16 (40.0)	32 (80.0)	8 (20.0)	0.043 (2.7)
French	35 (53.8)	26 (40.0)	4 (6.2)	46 (70.8)	18 (27.7)	1 (1.5)	0.035	96 (73.8)	34 (26.2)	110 (84.6)	20 (15.4)	0.023* (1.95)
Austrian	11 (50.0)	10 (45.5)	1 (4.5)	10 (45.5)	11 (50.0)	1 (4.5)	NS	32 (72.7)	12 (27.3)	31 (70.4)	13 (29.6)	NS
Slovenian	1	0	0	1	0	0	—	2	0	2	0	—
Total	125 (57.0)	83 (38.0)	11 (5.0)	148 (67.6)	63 (28.8)	8 (3.6)	0.015*	333 (76.0)	105 (24.0)	359 (82.0)	79 (18.0)	0.019 (1.4)*

*P-values statistically significant after Bonferroni correction ($P \leq 0.025$).
[a]Comparison of genotype frequencies by the haplotype relative risk test (HRR).
[b]Comparison of allele frequencies by the haplotype-based haplotype relative risk approach (HHRR).
[c]'Virtual' controls derived from nontransmitted alleles to the affected offspring.

Table 2 Genotypic and allelic distribution of the Val66Met SNP of the *BDNF* gene in patients with binge-eating/purging AN and 'virtual' controls

	Binge-eating/purging AN											
	n *Genotypes (%)*[a]							n *Alleles (%)*[b]				
	Probands			*Pseudocontrols*[c]			P-*value*	*Probands*		*Pseudocontrols*[c]		P-*value (OR)*
	ValVal	*ValMet*	*MetMet*	*ValVal*	*ValMet*	*MetMet*		*Val*	*Met*	*Val*	*Met*	
Italian	9 (56.2)	7 (43.8)	0	6 (37.5)	9 (56.2)	1 (6.25)	NS	25 (78.1)	7 (21.9)	21 (65.6)	11 (34.4)	NS
German	12 (85.7)	2 (14.2)	0	5 (35.7)	7 (50.0)	2 (14.2)	0.009*	26 (92.8)	2 (7.1)	17 (60.7)	11 (39.3)	0.0048* (8.4)
British	7 (53.8)	6 (46.2)	0	8 (61.5)	4 (30.8)	1 (7.7)	NS	20 (76.9)	6 (23.0)	20 (76.9)	6 (23.1)	NS
Spanish	7 (53.8)	6 (46.2)	0	8 (61.5)	4 (30.8)	1 (7.7)	NS	20 (76.9)	6 (23.0)	20 (76.9)	6 (23.1)	NS
French	35 (53.0)	27 (40.9)	4 (6.1)	40 (60.6)	23 (34.8)	3 (4.5)	NS	97 (73.5)	35 (26.5)	103 (78.0)	29 (22.0)	NS
Austrian	12 (92.3)	1 (7.7)	0	10 (76.9)	3 (23.1)	0	NS	25 (96.2)	1 (3.8)	23 (88.5)	3 (11.5)	NS
Slovenian	1 (20.0)	4 (80.0)	0	2 (40.0)	3 (60.0)	0	—	6 (60.0)	4 (40.0)	7 (70.0)	3 (30.0)	—
Total	83 (59.2)	53 (37.9)	4 (2.9)	79 (56.4)	53 (37.9)	8 (5.7)		219 (78.2)	61 (21.8)	211 (75.3)	69 (24.6)	NS

*P-values statistically significant after Bonferroni correction ($P \leq 0.025$).
[a]Comparison of genotype frequencies by the haplotype relative risk test (HRR).
[b]Comparison of allele frequencies by the haplotype-based haplotype relative risk approach (HHRR).
[c]'Virtual' controls derived from nontransmitted alleles to the affected offspring.

Table 3 TDT for the Val66Met and −270C/T SNPs of the *BDNF* gene in 158 trios with restricting AN from eight different European centres

Polymorphism	*Allele*	*Restricting AN* *Transmitted/nontransmitted*[a]	χ^{2b}	P-*value*
Val66Met	Val66	70/88	2.05	0.075
($n=158$)	Met66	88/70		
−270C>T	−270C	17/20	0.24	0.31
($n=37$)	−270T	20/17		
Haplotype		*Transmitted/nontransmitted*[a]	χ^{2b}	*P-value*
−270C-Met66		74/51	4.2	0.019 ++
−270C-Val66		63/87	3.8	0.025 −−
−270T-Val66		15/16	0.03	0.43
−270T-Met66		2/0	2.0	0.08

[a]Transmitted *vs* nontransmitted alleles from heterozygous parents.
[b]McNemar's χ^2.

Table 4 Q-TDT analysis of the Val66Met *BDNF* SNP and minimum BMI, maximum BMI and age at onset of weight loss (AO) in trios with restricting AN

ED-related trait	*Alleles*	*Restricting AN* T-*value*	P-*value*	*Direction of the association*
minBMI ($n=249$)	Val66	2.09	0.019	Positive
	Met66	−2.09		Negative
MaxBMI ($n=159$)	Val66	0.55	NS	—
	Met66	−0.55		—
AO ($n=222$)	Val66	−0.96	NS	—
	Met66	0.96		—

Thus, 343 parents heterozygous for the Val66Met SNP (158 ANR, 113 ANB and 72 BN) and 79 for the −270C/T SNPs (37 ANR, 24 ANB and 18 BN) were analysed by the TDT approach. No significant differences in the transmission of the *BDNF* alleles were observed when both polymorphisms were separately considered. However, the multiallelic version of the TDT revealed an excess of transmission of the −270C/Met66 haplotype (74 transmitted *vs* 51 not transmitted, $\chi^2=4.2$, $P=0.019$) and a reduced transmission of the −270C/Val66 haplotype (63 transmitted *vs* 87 not transmitted, $\chi^2=3.84$, $P=0.025$) to the affected ANR offspring (Table 3). No significant differences were observed when trios with ANB or BN were considered (data not shown).

The quantitative trait loci transmission disequilibrium test (Q-TDT) did not give evidence for the involvement of the −270C>T SNP in any of the ED-related traits. We neither observed association between the Val66Met variant and AO nor maxBMI, but, consistent with previously reported results,[10] we detected that the Met66 allele was transmitted with a lower minBMI than the Val66 allele ($T=2.09$, $P=0.019$, Table 4). After Bonferroni correction, considering two SNPs and three different ED-related phenotypes, these differences did not remain statistically significant.

Discussion

The results of the present study give additional evidence for the association between *BDNF* and the restrictive type of AN, replicating previous case–control studies by a family-based approach.[10–12] Our genetic data are in agreement with animal model studies where intraventricular administration of BDNF in rats induces starvation and weight loss. Accordingly, *BDNF* or its receptor *NTRK2* knockout mice develop obesity and hyperphagia, which also suggest the involvement of this neurotrophic factor in eating behaviour and body weight regulation.[5–9]

The HRR and HHRR analyses of family trios from eight different European centres showed that, over population heterogeneity, the Met66 allele of the Val66Met SNP is associated with ANR. By the TDT approach no differences were observed when both the −270C>T and Va166Met SNPs were separately considered, but we detected a preferential transmission of the −270C/Met66 haplotype to the affected ANR offspring.

Family-based association studies, which have similar sensitivity to case–control analyses when using the HRR test and the specificity of linkage analysis when considering the TDT method, are not sensitive to stratified populations and address the problem of stratification present in the population-based association approaches. However, the statistical power of the TDT method depends on the number of heterozygous parents analysed and becomes a critical issue in the study of susceptibility genes in a complex phenotype such as ED. Although our sample size is relatively large, the negative results of the TDT analysis, when both *BDNF* SNPs were separately analysed, should be considered with caution, as only 158 and 37 ANR trios were informative for the Val66Met and −270C/T SNPs, respectively, sample sizes that represent a statistical power of 77% for the Val66Met and 5.7% for the −270C>T SNPs.

On the other hand, the haplotype analysis considering both *BDNF* variants provided additional information, being the −270C/Met66 haplotype preferentially transmitted to the affected ANR probands. These results may reflect the positive association between the Met66 allele and ANR observed by the HRR and HHRR approaches. As this sequence variant consists of a functional amino-acid substitution that modulates the activity-dependent secretion of BDNF in hippocampal transfected neurons,[18] it could have direct biological consequences in the aetiology of ANR. Alternatively, a new variant in linkage disequilibrium with Met66 could be responsible for the genetic susceptibility to ED. Interestingly, another candidate gene involved in synapses, MALS3, is located 140 kb upstream of *BDNF* and its screening will provide further evidence for its possible involvement in the physiopathology of ED.[19,20]

We found no evidence for the significant role of *BDNF* in ANP or BN that was previously reported by a case–control study of six European populations.[11] Possible explanations for this discrepancy could be the presence of genetic heterogeneity, inadequate power of a small sample size once patients were subdivided according to the clinical subtype (statistical power <30% in all cases) or population structure effects. Moreover, the ANR sample may represent a more homogeneous clinical group that, in the presence of a moderate statistical power and genetic heterogeneity, may contribute to a better identification of the genetic factors involved. Alternatively, stratification bias, especially when analysing different populations, may have been a potential confounding factor in the previous case–-control association studies. However, although the contribution of the different centres with rather different sample sizes could increase the probability of type II errors, there are several evidences that suggest no stratification bias in the populations analysed: (a) we have analysed markers in other candidate genes for ED in the European samples participating in this study and no population differences among the different centres were detected,[21,22] (b) we have compared allelic and genotype frequencies of the *BDNF* SNPs among controls from the different centres and no significant differences have been observed and (c) Ardlie *et al*[23] analysed four different samples to detect the presence of population subdivision and suggested that carefully matched and moderate-sized samples from European populations are unlikely to contain levels of population stratification. All these evidences support the fact that the presence of population stratification among the different European centres participating in this study is unlikely to be involved in our positive results.

Although the number of ED trios was modest, once we subdivided the samples according to centres, the association between ANB and the Val66 allele in the German group suggests that, as previously reported, this population displays a different genetic background in comparison to the other European samples.[11] Alternatively, a differential Val66Met effect on ANR and ANB is also possible, but previous case–control studies argue against this hypothesis as the Met66 allele was associated to ANR, ANB and BN.[11,12] On the other hand, we did not find the previously reported association between the −270C/T SNP and BN or AO,[11] but its low heterozygosity in the studied groups or the presence of population heterogeneity could contribute to these negative results.

Under the hypothesis that AN and BN may share some aetiological basis,[15,24] we also focused our study on different ED phenotypical markers and found an association between the Met66 *BDNF* allele and low minBMI. These results are consistent with previous studies and suggest a role of this *BDNF* variant in the severity of the illness.[10]

In conclusion, the results of this family-based study give evidence for a *BDNF* contribution in the vulnerability to both ANR and minBMI in a sample of eight different European populations. This is the first family approach that replicates previous case–control studies reporting a positive association between *BDNF* and ED. However, it remains uncertain if the −270C/T SNP participates in eating behaviour and body weight regulation. Further studies in larger samples should provide additional clues about its role in the susceptibility to ED. Moreover, the characterization of additional SNPs within and flanking the *BDNF* gene, together with the analysis of other populations, may improve our understanding about the involvement of this neurotrophin in the vulnerability to AN and BN.

Subjects and methods

Study subjects

Family-based study A total of 453 family trios with ED 79.2% AN trios ($N=359$) and 20.8% BN trios ($N=94$)) were recruited from eight different European centres participating in the EC Framework V 'Factors in Healthy Eating'

Table 5 Distribution of 453 ED trios from eight European centres according to population and diagnosis

Population	*ANR trios*	*ANB trios*	*BN trios*
Italian – Milan	18	9	27
Italian – Florence	3	7	3
German	64	14	17
British	26	13	—
Spanish	20	13	34
French	65	66	—
Austrian	22	13	8
Slovenian	1	5	5
Total	219	140	94

QLK1-1999-916 consortium (France ($N=131$), UK ($N=39$), Germany ($N=95$), Milan ($N=54$), Florence ($N=13$), Spain ($N=67$), Austria ($N=43$) and Slovenia ($N=11$)). AN trios were subdivided in restricting ($N=219$) and binge-eating/purging ($N=140$) subtype. All probands included in this study had a minimum duration of the illness of 3 years to be considered as ANR or ANP. Table 5 shows the distribution of the ED family trios according to diagnosis and population. All probands fulfilled the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria for ED and were diagnosed using various types of interview according to the country. The Spanish and Florence samples were diagnosed with the Structured Clinical Interview for Mental Disorders, research version 2.0 (SCID-I), the British and Austrian samples with the ATE EAT, the French patients with the Diagnostic Interview for Genetic Studies (DIGS), the Italian sample from Milan with the Diagnostic Interview Schedule-Revised (DIS-R), and the German and Slovene samples with the Composite International Diagnostic Interview (CIDI). Physicians reached a consensus on the different interviews used for the diagnosis. Most of the patients were female ($N=441$, 97.3%) and DNA samples were used in previous association studies.[10,11,21,22] Probands of 339 ED trios (88%) were previously analysed in a case–control study to determine the *BDNF* participation in ED.[11] Clinical information was available from the majority of patients. The average age at assessment was 20.8 years old (SD = 6.7) for AN patients and 23.7 years old (SD = 5.3) for BN patients. The lifetime minBMI was 13.66 kg/m^2 (SD = 2.1) for AN patients and 18.63 kg/m^2 (SD = 3.0) for BN patients. The lifetime maxBMI was 20.53 kg/m^2 (SD = 3.7) for AN patients and 25.32 kg/m^2 (SD = 4.7) for BN patients. The study was approved by the Ethics Committee of each institution. Written informed consent was obtained from all subjects who participated in the study.

Molecular analysis

Genotyping of the −270C/T polymorphism located in the 5′-untranslated region of the *BDNF* gene and the Val66Met variant within the prodomain of the BDNF precursor was performed as previously described.[11]

Statistical analysis

Average minBMI, maxBMI, age of assessment and AO in each studied group were measured by the statistical package SPSS 10.0. The distribution of genotypes for the different populations was tested for the Hardy–Weinberg equilibrium by a χ^2 analysis using the INSTAT Graphpad software. Under the hypothesis that *BDNF* may confer susceptibility to the different ED subtypes in different ways and to reduce heterogeneity, patients were subgrouped according to the clinical subtypes of ANR, ANP and BN. The Italian samples from Milan and Florence were analysed together in all statistical tests. Linkage disequilibrium tests were performed in probands and their parents using the Haploview v.2.03 (http://www.broad.mit.edu/personal/jcbarret/haploview). The power analysis was performed *post hoc* on the ED groups with the Power Calculator software for the HRR analysis (Department of Statistics of the University of Los Angeles; http://calculators.stat.ucla.edu/powercalc) and with the Genetic Power Calculator for the TDT approach (http://statgen.iop.kcl.ac.uk/gpc;[25]). The transmitted and not transmitted genotypes from parents to the affected offspring were compared by the HRR strategy using the UNPHASED v.2.4 software.[26] The comparison of the transmitted and not transmitted alleles from parents to the child was assessed by the HHRR analysis [27] using a Fisher-exact test by the INSTAT Graphpad software. After Bonferroni correction, conisdering two different *BDNF* SNPs, significance was set up at P-values < 0.025. The TDT and the two-locus TDT were performed using McNemar's χ^2 by the GENEHUNTER program (version 1.0; 13). The analysis of the ED-related quantitative traits was performed in the overall sample of ED patients by the quantitative trait loci QTDT.[28] As the aim of this study was to replicate a previous reported association, we considered one-tailed P-values in all statistical tests.

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