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Mutation Screen of the Brain Derived Neurotrophic Factor Gene (*BDNF*): Identification of Several Genetic Variants and Association Studies in Patients With Obesity, Eating Disorders, and Attention-Deficit/Hyperactivity Disorder

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Several lines of evidence indicate an involvement of brain derived neurotrophic factor (BDNF) in body weight regulation and activity: heterozygous *Bdnf* knockout mice (*Bdnf*$^{+/-}$) are hyperphagic, obese, and hyperactive; furthermore, central infusion of BDNF leads to severe, dose-dependent appetite suppression and weight loss in rats. We searched for the role of *BDNF* variants in obesity, eating disorders, and attention-deficit/hyperactivity disorder (ADHD). A mutation screen (SSCP and DHPLC) of the translated region of *BDNF* in 183 extremely obese children and adolescents and 187 underweight students was performed. Additionally, we genotyped two common polymorphisms (rs6265: p.V66M; c.−46C > T) in 118 patients with anorexia nervosa, 80 patients with bulimia nervosa, 88 patients with ADHD, and 96 normal weight controls. Three rare variants (c.5C > T: p.T2I; c.273G > A; c.*137A > G) and the known polymorphism (p.V66M) were identified. A role of the I2 allele in the etiology of obesity cannot be excluded. We found no association between p.V66M or the additionally genotyped variant c.−46C > T and obesity, ADHD or eating disorders. This article contains supplementary material, which may be viewed at the American Journal of Medical Genetics website at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html.

KEY WORDS: weight regulation; BMI; anorexia nervosa; bulimia nervosa

INTRODUCTION

BDNF plays a key role in regulating neuronal survival during the development of the central nervous system, differentiation and maintenance of the phenotype of mature neurons [Maisonpierre et al., 1991], and prevents neuronal death [Tuszynski et al., 1994]. Human *BDNF* is localized on chromosome 11p14.1 (http://genome.ucsc.edu/, Freeze July 2003) and encodes a 247 amino acid (aa) preprotein that is proteolytically cleaved to form the 120 aa mature protein [Darling et al., 1983], which is 100% conserved between mouse, rat, pig, and humans [Maisonpierre et al., 1991]. Human *BDNF* consists of five alternatively used 5′ exons and one major 3′ exon. Alternative splicing of the 5′ exons results in six different transcripts leading to three preproprotein isoforms (a, b and c) that differ in the length of their signal peptide (http://www.ncbi.nlm.nih.gov/LocusLink, April 2004). Isoforms b and c contain additional N-terminal aa compared to isoform a.

Several lines of evidence indicate an involvement of genetic factors in the etiology of the complex and multifactorial disorders obesity, anorexia nervosa (AN), bulimia nervosa (BN), and attention-deficit/hyperactivity disorder (ADHD). We propose a role of *BDNF* in the development of these disorders for the following reasons: (i) obesity and eating disorders: heterozygous *Bdnf* knockout mice (*Bdnf*$^{+/-}$) are obese and develop hyperphagia [Kernie et al., 2000]. Their increase in body weight is similar to that seen in heterozygous melanocortin-4-receptor deficient (*Mc4r*$^{+/-}$) mice, a well-known model for human obesity [e.g., Huszar et al., 1997; Hinney et al., 2003]. BDNF is expressed at high levels in the ventromedial hypothalamus (VMH), where it is regulated by nutritional state and by MC4R signaling [Xu et al., 2003]. Bilateral lesions of the VMH entail hyperphagia and obesity [Shimizu et al., 1987]. Central infusion of BDNF leads to severe, dose-dependent appetite suppression, weight loss, and increase in hypothalamic 5-hydroxy-indoleacetic acid (5-HIAA) and serotonin in rats, implying an anorexigenic function of BDNF [Pelleymounter et al., 1995].

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A recent study revealed a strong association of the M66 allele of *BDNF* with obsessive-compulsive disorder [OCD; Hall et al., 2003], which is quite common in patients with eating disorders, suggesting that there may be a common genetic predisposition to both OCD and AN [Halmi et al., 1991]. Ribases et al. [2003] recently detected an association of the M66 variant in the region encoding BDNF proprotein [Momose et al., 2002] to AN (restricting type) and to a low minimum body mass index (MBMI). Additionally, it was shown that female patients with eating disorders (ED) have decreased levels of serum BDNF compared to healthy normal weight controls [Nakazato et al., 2003]. (ii) *ADHD*: a relationship between ADHD and BDNF has already been hypothesized [Tsai, 2003]. Conditional deletion of *Bdnf* in postnatal mice brain leads to hyperactivity after exposure to stressors [Rios et al., 2001]. A recent study revealed evidence for the involvement of the M66 variant (rs6265) of BDNF in poor verbal episodic memory [Egan et al., 2003]. Therefore, the M66 allele might be relevant in ADHD. BDNF affects central nervous system myelination [Cellerino et al., 1997]; central dysmyelination has been found in patients with ADHD [Overmeyer et al., 2001]. Neurochemical and behavioral analysis of heterozygous $Bdnf^{+/-}$ mice revealed that a partial impairment of BDNF expression causes physiological disturbances which were associated with impaired impulse control, manifested as exaggerated aggressiveness, and excessive appetite/food intake [Lyons et al., 1999].

Therefore, we hypothesize that gain of function mutations could predispose to AN, whereas loss of function mutations could be expected to result in obesity and ADHD. The M66 variant is of particular interest, because it is the only known frequent non-conservative polymorphism in the *BDNF* gene. Furthermore, the M66 variant affects intracellular processing and secretion of the mature protein [Egan et al., 2003].

MATERIALS AND METHODS

In order to assess an involvement of *BDNF* in weight regulation, we screened the translated region of *BDNF* in 183 extremely obese children and adolescents and 187 healthy underweight students (initial screening sample). We identified two new variants and three known SNPs, which enabled us to perform association studies.

Study Subjects

We screened 183 extremely obese children and adolescents and 187 healthy underweight students. The mean BMI percentile of the 183 obese probands exceeded the 99th BMI-percentile, the BMI of the underweight students was below the 15th percentile, as previously determined in a representative German population sample [Hebebrand et al., 1996]. For association studies, we used the initial screening-sample and samples of patients with ADHD, AN, BN, and normal weight controls (see the online Table II at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html). Patients with ED or with ADHD fulfilled DSM-IV criteria [APA, 1994]. Written informed consent was given by all participants and, in the case of minors, their parents. This study was approved by the Ethics Committee of the University of Marburg.

PCR, DHPLC, and SSCP

Six transcript variants encoding three preproprotein isoforms have been described for this gene. The nomenclature of the described polymorphisms is according to den Dunnen and Antonarakis [2001] and in relation to transcript variant 1 encoding isoform a (Acc. No. NM_001709). Variant c.−46C > T was earlier described as 270C > T [Kunugi et al., 2001]. We screened the translated region of human *BDNF* in two overlapping fragments A and B (see the online Table III at http://www.interscience.wiley.com/jpages/0148-7299/suppmat/index.html). For PCR amplification primers were placed so that potential splice site variants could be detected. Mutation screen on fragment A was performed with denaturing high performance liquid chromatography (DHPLC) analysis on Transgenomic WAVE® system [Transgenomic, Cheshire, UK; Oefner and Underhill, 1998]. All chromatograms were compared with chromatograms of sequenced wild-type samples. PCR amplicons that showed a peak appearance different to the wild-type pattern were sequenced (Seq Lab, Göttingen, Germany). To detect mutations in fragment B, the PCR-products were digested and standard nonisotopic single-strand conformation polymorphism analyses (SSCP) were performed at room temperature and at 4°C [Hinney et al., 1999]. The sensitivities for DHPLC have been described to be approximately 95% [Ellis et al., 2000] and about 97% for SSCP by using two temperatures, respectively [Salazar et al., 2002].

SNPs

PCR products of all SNPs were run on ethidium bromide-stained 2.5% agarose gels. Positive controls for the variant alleles were run on each gel. For validity of the genotypes, allele determinations were rated independently by at least two experienced individuals. Discrepancies were resolved unambiguously either by reaching consensus or by retyping (see the online Table IV at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html).

Statistics

Differences in genotype frequencies were investigated using the Cochran-Armitage trend test. Pearson's χ^2-tests were carried out to investigate differences in allele frequencies. Initially, obese children and adolescents were compared with underweight students. Our latter analyses tested each of the groups AN, BN, and ADHD separately against normal weight students. We did not correct for the multiple tests we performed for the different groups at the two loci. Therefore, all reported *P* values are nominal.

RESULTS

By sequencing PCR products showing an aberrant SSCP or DHPLC-pattern we identified three rare variants (c.5C > T; c.273G > A; c.*137A > G) in addition to the common missense mutation p.V66M. In the study groups comprising 183 extremely obese children and adolescents and 187 healthy underweight students each rare variant was observed only once: (a) the novel silent variant c.273G > A in codon 91 of the region coding for the proprotein was discovered by DHPLC. An extremely obese male (age 16.2 years, BMI 50.4 kg/m^2, BMI ≥ 99th percentile) was heterozygous for this variant. (b) A known variant c.5C > T (rs8192466), previously detected in a patient with idiopathic congenital central hypoventilation syndrome [CCHS; Weese-Mayer et al., 2002] leading to the non-conservative non-synonymous aa change p.T2I was detected in a single extremely obese boy (age 11.1 years, BMI 40.4 kg/m^2, BMI ≥ 99th percentile) by DHPLC. The allele coding for I2 was transmitted by the overweight mother (BMI 28.7 kg/m^2); the overweight sib had inherited the wild type allele. (c) One of the underweight controls (age 24.1 years, BMI 19.7 kg/m^2, 6th BMI percentile) was heterozygous for a novel 3′UTR variant c.*137A > G, detected by SSCP.

We analyzed two common polymorphisms (V66M in the 5′ pro-region; SNP c.−46C > T in one of the 5′UTR exons, Kunugi et al., 2001) in the initial study groups and additionally in 118 patients with AN, 80 patients with BN, 88 patients with

TABLE I. Genotype Distribution of p.V66M (Isoform a, rs6265) in the Translated Region and SNP c.−46 > T in the Untranslated Region of *BDNF*

	Obese children and adolescens, n (%)	Underweight controls, n (%)	Normal weight controls, n (%)	Patients with AN, n (%)	Patients with BN, n (%)	Patients with ADHD, n (%)
V66M						
n	183	187	96	118	80	83
GG	114 (62.3)	110 (58.8)	62 (64.6)	81 (68.6)	51 (63.8)	56 (67.5)
GA	57 (31.1)	71 (38.0)	33 (34.4)	32 (27.1)	27 (33.7)	24 (28.9)
AA	12 (6.6)	6 (3.2)	1 (1.0)	5 (4.3)	2 (2.5)	3 (3.6)
c.−46 > T						
n	182	185	82	118	80	86
CC	158 (86.8)	164 (88.7)	75 (91.5)	103 (87.3)	65 (81.3)	75 (87.2)
CT	24 (13.2)	20 (10.8)	7 (8.5)	15 (12.7)	13 (16.2)	10 (11.6)
TT	0 (0)	1 (0.5)	0 (0)	0 (0)	2 (2.5)	1 (1.2)

AN, anorexia nervosa; BN, bulimia nervosa; ADHD, attention-deficit/hyperactivity disorder.
Genotype-frequencies did not differ from Hardy–Weinberg equilibrium.

ADHD, and 96 normal weight controls. Genotype frequencies did not differ from Hardy–Weinberg equilibrium. Association studies revealed no significant differences in genotype or allele distributions between extremely obese children and adolescents and underweight controls, as well as between AN, ADHD, and normal weight students; all nominal P values were >0.2 (Table I). We detected a trend towards association for the −46T allele in 80 patients with BN compared to 82 normal weight controls (nominal $P = 0.06$ for the genotypes and nominal $P = 0.03$ for the alleles). At the nominal significance level of 5%, for the given sample sizes and the observed allele frequencies in controls, our study had a power of 80% to detect a 10% increase in M66 frequency between underweight controls and obese children and adolescents; for the comparisons involving normal weight controls the respective power was about 60%. For c.−46C > T, we had a power of 80% to detect a twofold increase in −46T allele frequency between underweight controls and obese children and adolescents; for the comparisons involving normal weight controls the respective power was about 40%.

DISCUSSION

We screened the translated main exon of *BDNF* for mutations in a total of 370 German obese and underweight individuals. Three variants were identified apart from the previously known SNP p.V66M (rs6265): (i) we found the previously detected non-conservative amino acid substitution p.T2I [Weese-Mayer et al., 2002] in a single extremely obese male who inherited the mutation from his obese mother. Amino acid position 2 of isoform a is equivalent to position 10 in isoform b and position 17 in isoform c of the BDNF preproprotein. The threonin at this position is conserved between all species, of which a sequence has been deposited into public databases, including mouse, rat, pig, various bears, kangaroo, chicken, carp, platy fish, and zebra fish [Weese-Mayer et al., 2002]. It is unclear whether the I2 variant affects the mode of action of the signal peptide. If it results in a loss of function, the mutation could very well be relevant for obesity; the body weights of the extremely obese carrier (BMI 40.4 kg/m^2) of the I2 variant and his overweight mother are in the expected range as based on the phenotype of *Bdnf*$^{+/-}$ mice that show a significant weight increase in males (50%) and females (27%) compared to wild type littermates. The weight of the index mutation carrier has increased by approximately 50 kg in the last 3 years after loss of 15 kg in an inpatient weight reduction program. Initially, the p.T2I has been described in a dysphagic patient affected by CCHS with a BMI of 16 [Weese-Mayer et al., 2002; Weese-Mayer, personal communication]. His heterozygous father has a BMI of 26 and does not show symptoms of CCHS, but of the associated autonomic nervous system dysfunction [ANSD; Weese-Mayer et al., 2002; Weese-Mayer, personal communication]. This is not readily compatible with a putative role of I2 in the development of obesity. Nevertheless, I2 could be involved in the etiology of obesity because being affected with CCHS or ANSD could explain why obesity does not ensue in these two heterozygotes. ANSD and CCHS are severe syndromes accompanied by oesophageal dysmotility, gastroesophageal reflux, and dysphagia. Until further functional studies are carried out, it is unclear what effect the mutation at I2 might have on the mode of action of the signal peptide and how it may relate to the clinical condition of obesity. (ii) The novel variant c.273G > A was detected once in an extremely obese male. We assume that there is no major effect because this mutation is silent. (iii) The 3′UTR variant c.*137A > G was detected in one underweight control (BMI 19.7 kg/m^2), an influence on the mode of action of BDNF is unlikely. (iv) We did not detect an association between obesity, AN or ADHD and SNP p.V66M or c.−46C > T in the genomic region of *BDNF*. For BN, we found a trend towards an association with −46T. We were not able to follow-up on this result due to our limited number of BN cases and the trend needs to be judged in the context of the multiple tests we performed. Apart from a false positive result, two different mechanisms could explain this finding: First, the c.−46C > T variant is in linkage disequilibrium with a yet unknown variant or an unknown susceptibility gene directly involved in the etiology of BN. Alternatively, this variant itself entails an increased risk that may result from an alteration in the translation efficacy [Shintani et al., 1992]. No data are available as to potential functional consequences of this variant. Our results were not in line with Ribases et al. [2003], who reported an association of the M66-allele with AN in a Spanish sample. Some of our data on patients with AN or BN and controls have been included in a recent meta-analysis pertaining to the polymorphisms V66M and c.−46C > T. The meta-analysis showed that the M66 variant is strongly associated to all ED subtypes and that the −270C (−46T) BDNF variant has an effect on BN and age at onset of weight loss [Ribases et al., 2004].

In conclusion, our results do not suggest a large role of genetic variation of *BDNF* in AN, BN, ADHD, or obesity; possibly the I2 variant plays a role in obesity. To exclude moderate effects of the two investigated polymorphisms larger samples need to be assessed.

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