

ORIGINAL ARTICLE

Evidence for three genetic loci involved in both anorexia nervosa risk and variation of body mass index

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The maintenance of normal body weight is disrupted in patients with anorexia nervosa (AN) for prolonged periods of time. Prior to the onset of AN, premorbid body mass index (BMI) spans the entire range from underweight to obese. After recovery, patients have reduced rates of overweight and obesity. As such, loci involved in body weight regulation may also be relevant for AN and vice versa. Our primary analysis comprised a cross-trait analysis of the 1000 single-nucleotide polymorphisms (SNPs) with the lowest *P*-values in a genome-wide association meta-analysis (GWAMA) of AN (GCAN) for evidence of association in the largest published GWAMA for BMI (GIANT). Subsequently we performed sex-stratified analyses for these 1000 SNPs. Functional *ex vivo* studies on four genes ensued. Lastly, a look-up of GWAMA-derived BMI-related loci was performed in the AN GWAMA. We detected significant associations (*P*-values < 5×10^{-5} , Bonferroni-corrected *P* < 0.05) for nine SNP alleles at three independent loci. Interestingly, all AN susceptibility alleles were consistently associated with increased BMI. None of the genes (chr. 10: *CTBP2*, chr. 19: *CCNE1*, chr. 2: *CARF* and *NBEAL1*; the latter is a region with high linkage disequilibrium) nearest to these SNPs has previously been associated with AN or obesity. Sex-stratified analyses revealed that the strongest BMI signal originated predominantly from females (chr. 10 rs1561589; $P_{\text{overall}}: 2.47 \times 10^{-66}/P_{\text{females}}: 3.45 \times 10^{-67}/P_{\text{males}}: 0.043$). Functional *ex vivo* studies in mice revealed reduced hypothalamic expression of *Ctbp2* and *Nbeal1* after fasting. Hypothalamic expression of *Ctbp2* was increased in diet-induced obese (DIO) mice as compared with age-matched lean controls. We observed no evidence for associations for the look-up of BMI-related loci in the AN GWAMA. A cross-trait analysis of AN and BMI loci revealed variants at three chromosomal loci with potential joint impact. The chromosome 10 locus is particularly promising given that the association with obesity was primarily driven by females. In addition, the detected altered hypothalamic expression patterns of *Ctbp2* and *Nbeal1* as a result of fasting and DIO implicate these genes in weight regulation.

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INTRODUCTION

The joint analysis of genome-wide association studies (GWAS) data pertaining to different phenotypes/diseases with overlapping

or co-morbid endophenotypes recently led to the discovery of novel genes that had escaped detection in single phenotype/disease analyses. For instance, by a cross-disorder analysis of five

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major psychiatric disorders common underlying biological mechanisms were revealed.^{1,2} A number of genetic variants were associated with more than one psychiatric disorder, illustrating the usefulness of the approach. Other cross-disorder analyses have shown overlapping genetic risk factors for phenotypes that had not been expected to share risk factors (e.g., ulcerative colitis and bone density or white blood cell count³). Heritability of anorexia nervosa (AN) is moderately high.^{4–8} However, the two published genome-wide association meta-analysis (GWAMA)^{9,10} were underpowered to detect signals of small effect sizes, which are characteristic of single-nucleotide polymorphisms (SNPs) identified for other psychiatric disorders.^{1,2} The largest GWAMA for AN was performed in 2907 patients with AN and 14,860 controls by the Genetic Consortium for AN (GCAN) and the Wellcome Trust Case Control Consortium 3 (WTCCC3). Although a global meta-analysis comprised discovery and replication data sets on a total of 5551 AN cases and 21,080 controls, genome-wide significance was not reached.¹⁰ However, 76% of the variant effects were directionally consistent between discovery and replication groups. This observation was unlikely to be spurious ($P = 4 \times 10^{-6}$).¹⁰

A substantial genetic contribution to the variance of body mass index (BMI) is implicated by twin, family and adoption studies.^{11,12} The largest currently published GWAMA pertaining to BMI variance revealed 97 genome-wide significant ($P \leq 5 \times 10^{-8}$) gene loci;¹³ we use the term 'BMI SNPs' for those SNPs associated with an increased BMI. As most of the respective genes are expressed in the brain, a largely central regulation of human body weight appears likely.^{13,14} A region on chromosome 16p11.2 supports a possible genetic link between obesity and AN. Carriers of the respective deletion(s) are hyperphagic and obese, whereas the carriers of the duplication(s) are underweight and show restrictive/selective eating behavior.^{15,16}

Sex-specific analyses have previously been conducted for BMI and related phenotypes. For instance, the weight increasing effect was more pronounced in female mice of the initial melanocortin-4 receptor gene (*Mcr4*) knock-out strain.¹⁷ In humans with *MC4R* mutations leading to reduced function, the weight increasing effect was also stronger in females.¹⁸ Sex-stratified GWAMAs for waist-hip ratio (WHR) variation and other anthropometric traits (height, weight, BMI, waist circumference and hip circumference) revealed a sexual dimorphism in the genetic effects for fat distribution and waist phenotypes.^{19–22} For many of these, genome-wide significance was detected for females only.^{19,20}

AN might be considered as an extreme weight condition,²³ potentially entailing that genetic factors involved in body weight regulation may overlap with those predisposing to AN as suggested by several groups.^{8,10,23–30} Recent LD-score regression analyses revealed a negative genetic correlation between AN and obesity (and a similar genetic correlation with BMI), suggesting that the same genetic factors influence normal variation in BMI as well as dysregulated BMI in AN.³⁰ However, in the latest GWAMA for AN 89 SNPs with genome-wide significance for BMI variation and obesity^{31,32} and 15 SNPs related to extreme obesity³¹ were not associated with AN.¹⁰

There is no evidence for an aberrant body weight regulation prior to manifestation of AN; thus, recalled premorbid weight of AN patients seemingly covers the whole BMI range.^{33–35} The BMI range of patients at medium term (5–10 years) follow-ups is shifted to the left (lower BMI); in recovered patients overweight occurs with a substantially lower probability than in the general population.^{36,37}

Here we performed three cross-trait analyses involving AN risk and BMI variation in two GWAMAs. First, we performed a cross-trait analysis of the 1000 SNPs with the lowest *P*-values from the largest GWAMA for AN (GCAN¹⁰) for evidence of association in the largest published GWAMA for BMI variation (GIANT¹³). Second, we performed sensitivity analyses in sex-stratified data sets from the BMI GWAMA for the best cross-trait SNPs (Table 1) because of the

profound female preponderance in AN;^{38,39} furthermore, sex-stratified analyses have revealed BMI loci that had not been detected in sex-combined analyses.¹³ Finally, we performed a look-up of GWAMA-derived BMI, (childhood) obesity and WHR loci within the AN GWAMA.

Post hoc we also performed (1) a look-up of the best cross-trait SNPs (Table 1) in: (1) obese children and adolescents from the EGG Consortium,⁴⁰ and (2) the first GWAS for AN⁹ comprising 1,033 AN cases and 3,733 pediatric controls from the Price Foundation Collaborative Group and the Children's Hospital of Pennsylvania. Finally, we performed functional studies of the four genes nearest to the best cross-trait findings.

MATERIALS AND METHODS

Look-up of 'AN SNPs' (GCAN) in GIANT GWAMA for BMI including sex-specific analyses

Our primary analysis is based on the *in silico* look-up of the 1000 best hits according to *P*-value (SNPs in high linkage disequilibrium (LD) were not excluded) derived from the case-control AN GWAMA¹⁰ in the large-scale GWAMA of up to 322,135 individuals from the population-based GIANT meta-analysis for BMI.¹³ In light of the aforementioned results for obesity risk alleles in the AN GWAMA,¹⁰ we did not pursue the directional hypothesis that AN susceptibility/risk alleles are protective of obesity (i.e. are expected to be BMI lowering); as a consequence, we report two-sided tests. Secondly, we performed sex-stratified analyses for the best cross-trait SNPs in the BMI GWAMA.¹³

We estimated the percentage of AN GWAMA SNPs that met the same *P*-value threshold in the BMI GWAMA (Supplementary Figure S1). We estimated that the genetic overlap of BMI and AN can seemingly be demonstrated if the number of SNPs analyzed is larger than 500, so that the 1000 SNPs we had chosen is justified. We decided against a computational derivation of an 'optimal' cutoff as this could inflate the type I error rate. We did not aim for a comprehensive assessment of the joint common SNP variation architecture of both traits.

Post hoc we performed analyses in the sub data sets of GIANT (a: full GWAS chip data on $N \sim 233,000$; b: MetaboChip on $N \sim 88,000$) to analyze if the observed effects are confirmed for each sub data set.

Look-up of 'BMI SNPs' in GWAMA for AN susceptibility (GCAN)

We performed an *in silico* look-up of the 56 novel genome-wide significant 'BMI SNPs' detected by Locke *et al.*¹³ in the case-control AN GWAMA (GCAN¹⁰). Subsequently we also analyzed previously described SNPs for BMI, obesity, childhood obesity and WHR (see Supplementary Tables). A total of 2916 quality-controlled genotypes of controls were included in both GCAN and GIANT ($n = 1437$ NBS-WTCCC National Blood Service donors and $n = 1479$ British 1958 birth cohort-WTCCC). Balancing between consistency (i.e. running our analyses on the same data sets as those published) and the necessity of sample independence, we rendered a re-analysis excluding these overlapping samples unnecessary.

Subsequent look-ups in independent GWAS data sets

We performed a look-up of the best cross-trait SNPs in GWAMA data of the EGG Consortium⁴⁰ consisting of 5530 obese children and adolescents (BMI ≥ 95 th percentile) and 8318 controls (BMI < 50 th percentile). Data on the childhood obesity trait has been contributed by the EGG Consortium and was downloaded from www.egg-consortium.org.⁴⁰ An additional look-up of the best cross-trait SNPs from GCAN and GIANT was performed in the first GWAS for AN⁹ consisting of 1033 AN cases and 3733 pediatric control subjects of European ancestry; five SNPs were available.

Written informed consent to take part in genetic association studies was given by all participants and in case of minors by their parents. Studies were approved by the respective institutional review boards or ethics committees and conducted in accordance with the Declaration of Helsinki.^{9,10,13,40}

Statistical analyses

We performed two main analyses and one stratified analysis nested within the first main analysis focussing on European-descent individuals of two GWAMAs. The GWAMA for AN¹⁰ was performed as fixed-effect meta-analysis based on single-SNP case-control association analyses under an

Table 1. Nine of the 1000 SNPs with the lowest *P*-values in a GWAS for AN risk (GCAN⁹) are associated with increased BMI (GIANT¹¹ with Bonferroni-corrected *P* < 0.05 significance; sorted according to the nominal *P*-values for increased BMI in all GIANT participants)

Chromosome/ position SNP nearest gene	Location	Rank in AN GWAS	AN effect allele/ frequency in AN cases	Odds ratio (s.e.)	<i>P</i> -value for AN risk	Frequency of AN reference allele for BMI	β (s.e.) for BMI for reference allele	Nominal <i>P</i> -value for increased BMI: all female/male	Bonferroni- corrected <i>P</i> -value ^a	Direction of effect ^b +/-
10/126685663 rs1561589 CTBP2	Intron	201	A/0.33	1.14 (0.04)	7.74 × 10 ⁻⁰⁵	A/0.34	0.0157 (0.0033)	2.47 × 10 ⁻⁰⁶ 3.45 × 10 ⁻⁰⁷ /0.043	0.0025	+
10/126681170 rs12771627 CTBP2	Intron	190	G/0.75	0.87 (0.03)	7.28 × 10 ⁻⁰⁵	G/0.74	-0.0162 (0.0035)	4.25 × 10 ⁻⁰⁶ 5.8 × 10 ⁻⁰⁶ /0.022	0.0043	+
10/126674064 rs11245456 CTBP2	Intron	177	C/0.75	0.87 (0.03)	6.79 × 10 ⁻⁰⁵	C/0.75	-0.0171 (0.0037)	4.58 × 10 ⁻⁰⁶ 1.03 × 10 ⁻⁰⁵ /0.009	0.0046	+
19/34978662 rs17513613 CCNE1	Distant 5'	409	T/0.70	0.88 (0.03)	0.0002	T/0.67	-0.015 (0.0033)	5.41 × 10 ⁻⁰⁶ 6.4 × 10 ⁻⁰³ / 1.24 × 10 ⁻⁰⁵	0.0054	+
2/203492447 rs17406900 CARF	Intron	709	A/0.48	0.90 (0.03)	0.0003	A/0.49	-0.0134 (0.0031)	1.08 × 10 ⁻⁰⁵ 1.8 × 10 ⁻⁰⁴ / 2.27 × 10 ⁻⁰³	0.0108	+
2/203639257 rs7593917 NBEAL1	Intron	444	A/0.46	0.89 (0.03)	0.0002	A/0.46	-0.0131 (0.0031)	2.48 × 10 ⁻⁰⁵ 9.54 × 10 ⁻⁰⁵ / 9.39 × 10 ⁻⁰³	0.0248	+
2/203582157 rs11691351 NBEAL1	Distant 5'	412	A/0.46	0.89 (0.03)	0.0002	A/0.46	-0.0126 (0.0031)	3.57 × 10 ⁻⁰⁵ 1.98 × 10 ⁻⁰⁴ / 8.21 × 10 ⁻⁰³	0.0357	+
19/34988693 rs8102137 CCNE1	Distant 5'	248	T/0.70	0.88 (0.03)	9.45 × 10 ⁻⁰⁵	T/0.67	-0.0169 (0.0041)	3.76 × 10 ⁻⁰⁵ 0.006/2.46 × 10 ⁻⁰⁴	0.0376	+
2/203635796 rs7573079 NBEAL1	Intron	401	G/0.46	0.89 (0.03)	0.0002	G/0.46	-0.0124 (0.0031)	4.61 × 10 ⁻⁰⁵ 2.80 × 10 ⁻⁰⁴ /0.008	0.0461	+

Abbreviations: BMI, body mass index; CARF, calcium responsive transcription factor; CCNE1, cyclin E1; CTBP2, C-terminal binding protein 2; GWAS, genome-wide association studies; NBEAL1, neurobeachin-like 1; SNP, single-nucleotide polymorphism. ^aPrimary analysis, sex-combined, correction for 1000 tests. ^bDirection of effect: + the effect/risk allele for increased BMI and AN risk are identical; - the effect/risk allele for increased BMI and AN risk are not identical.

additive genetic model with control for population stratification at the discovery data set level. Similarly, the GWAMA for BMI variation¹³ also worked with a fixed-effect meta-analysis based on discovery data set results obtained under a linear regression model adjusted for age, sex and study-specific covariates including control for population stratification effects. For the first main analysis, we looked-up the 1000 SNPs of the GWAMA for AN¹⁰ with the lowest *P*-values (discovery *P*-values from 5.56 × 10⁻²² to 4.79 × 10⁻⁴, of note: the SNP with the lowest *P*-value in the initial discovery GWAS for AN was not confirmed by genotyping in the replication sample¹⁰) in the GWAMA for BMI.¹³ We applied a conservative Bonferroni correction to the uncorrected *P*-values of the GWAMA for BMI to address multiple testing (see Table 1 and Supplementary Tables), and accordingly regarded all associations as significant which met a nominal *P*-value ≤ 5 × 10⁻⁵ (Table 1). For the SNPs with significant associations in the GWAMA for BMI, we also report the results of sex-stratified sensitivity analyses (Table 1). For the second main analysis, we performed a look-up of the 97 BMI loci in the GWAMA for AN. The direction of effect was evaluated only for SNPs with a nominal *P*-value ≤ 0.05.

Post hoc we also analyzed genome-wide significant loci for BMI, obesity, childhood obesity not originally described in Locke et al.¹³ (reviewed in Yazdi et al.⁴¹) and 68 genome-wide significant loci for WHR derived from a European GWAMA primary analysis (GIANT²¹) in the GWAMA for AN (GCAN¹⁰).

Animals and diet

Unless stated otherwise, male C57BL/6J mice were fed *ad libitum* with either a standard chow diet (Harlan Teklad LM-485; 5.6% kcal fat) or a high-fat diet (D12331; Research Diets, New Brunswick, NJ, USA; 58% kcal fat). The mice had free access to water and were maintained under constant ambient conditions (22 ± 1 °C, constant humidity, 12 h/12 h light/dark cycle). All animal studies were performed in Cincinnati, OH, USA and were approved by the Animal Ethics Committee of Cincinnati, OH, USA.

Gene expression analyses

To assess effects on fasting and re-feeding, hypothalamic gene expression was profiled in male 27/28-week-old C57BL/6J mice fed either *ad libitum*

with a regular chow diet, or which had been fasted for 12, 24 or 36 h, or which had been fasted for 36 h and then re-fed for 6 h using either a fat-free diet or a high-fat diet (*N* = 6–8 mice per group). The use of existing *ex vivo* material is in agreement with the US and German guidelines of the Animal Welfare Committee to restrict animal experiments to an absolutely necessary minimum. Target genes were amplified using the ViiA 7 real-time PCR system (Life Technologies, Darmstadt, Germany); results were normalized to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (HPRT). The used primer sequences were CTBP2-F: 3'-TACCACACCATCACCCTCAC-5'; CTBP2-R: 3'-TGTGGCAGACTGTGCAATCT-5'; CCNE1-F: 3'-AGCCTCGGAAAATCAGACCA-5'; CCNE1-R: 3'-CTTCGCACACCTCCATTAGC-5'; CARF-F: 3'-GTGGACGACAGATAGTGGGA-5'; CARF-R: 3'-GGAGA GGAGAGTCTTGGCTG-5'; NBEAL1-F: 3'-AGGAGAAGGAAATGGCTGATCA-5' and NBEAL1-R: 3'-TCCACTGTGAGAGAAGCTGG-5'. Data represent means ± s.e.m. **P* < 0.05, ***P* < 0.01, based on a one-way ANOVA with Dunnett's multiple comparison *post hoc* test.

To additionally assess the effects of a high-fat diet on hypothalamic expression, *Nbeal1* and *Ctbp2* was assessed in age-matched male C57BL/6J mice fed either a regular chow diet (body weight 32.69 ± 0.45 g) or a high-fat diet (body weight 54.72 ± 1.25 g; *N* = 7–8 mice per group). Data represent means ± s.e.m.

In silico analyses

Expression patterns and known variants in the coding regions (missense, nonsense and frameshift) were analyzed *in silico* (<http://www.genecards.org/>; <http://exac.broadinstitute.org/about>).

RESULTS

Association of AN risk SNPs with increased BMI

We detected association (*P*-values < 5 × 10⁻⁵, Bonferroni-corrected *P* < 0.05) at three independent chromosomal loci in the BMI GWAMA (chromosome 2: four SNPs in LD, *r*² ≥ 0.819, *D'* = 1; chromosome 10: three SNPs, *r*² ≥ 0.363, *D'* ≥ 0.728; and chromosome 19: two SNPs, *r*² = 1, *D'* = 1); the lowest *P*-value (rs1561589, 2.47 × 10⁻⁶, *P*_{corrected} = 0.0025) was observed at the chromosome

10 locus (Table 1). Within the GIANT¹³ data we *post hoc* also analyzed the data sets separately for (a) full GWAS chip data (HapMap imputed) on $N \sim 233,000$ and (b) Metabochip on $N \sim 88,000$ (Supplementary Table S1). Both independent data sets confirmed the association of the nine SNPs.

The nearest genes to these nine SNPs ordered from lowest to highest P -values are: (1) chromosome 10: *CTBP2* (C-terminal binding protein 2 gene); (2) chromosome 19: *CCNE1* (cyclin E1 gene); (3) chromosome 2: *CARF* (calcium responsive transcription factor gene) and (4) *NBEAL1* (neurobeachin-like 1 gene). The third chromosomal locus included two genes, because the four SNPs are located in a region with high LD (lowest LD for the four SNPs: $r^2 \geq 0.819$, $D' = 1$). Interestingly, for all SNPs, the AN risk alleles were consistently associated with increased BMI (Table 1).

Sex-specific analyses for the best cross-trait SNPs (Table 1) revealed that the chromosome 10 association signal was primarily driven by females. Again, *post hoc* sex-specific analyses in the sub data sets of GIANT (a: full GWAS chip data on $N \sim 233,000$; b: Metabochip on $N \sim 88,000$; Supplementary Table S1) confirmed the larger effect in females for the best locus.

Further look-ups

Because AN typically manifests during adolescence, we analyzed the identified SNPs in the EGG Consortium data set,⁴⁰ which includes only children and adolescents. The look-up of the nine cross-trait SNPs (Table 1) did not reveal significant findings at the five SNPs available (P -values from 0.0916 at rs11245456 to 0.6075 at rs1561589). However, the direction of effect was the same between AN risk and early onset extreme obesity in all five SNPs.

The look-up of the nine cross-trait SNPs in the first GWAS for AN⁹ comprising 1033 AN cases and 3733 pediatric controls (five SNPs were available, each locus was represented) showed nominally significant results for two SNPs at chromosome 2 (rs17406900, nominal $P = 0.03$; rs7573079, nominal $P = 0.04$), our second best locus. However, for these SNPs the direction of effect was opposite to the effect in GCAN.

Association of 'BMI SNPs' with AN

The look-up of the 'BMI SNPs' in the AN GWAMA did not reveal (Bonferroni-corrected for 97 SNPs) significant results (Supplementary Tables S2–S4). Similarly, *post hoc* look-ups of additional genome-wide significant loci for BMI, obesity, childhood obesity⁴¹ (Supplementary Table S5) and WHR²¹ (Supplementary Table S6) in the GWAMA for AN (GCAN¹⁰) did not reveal statistically significant findings after correction for multiple testing.

In silico analyses

All four genes located at the three identified loci are widely expressed in brain tissues, including the hypothalamus (<http://www.genecards.org/>). A spectrum of different, potentially functionally relevant variants (missense, nonsense and frameshift) was detected for all four genes (Supplementary Table S7).

Mouse model

Gene expression profiling of *Ctbp2*, *Ccne1*, *Carf*, *Nbeal1* in male C57BL/6J mice revealed that hypothalamic expression of both *Ctbp2* and *Nbeal1* was decreased by fasting (one-way ANOVA $P < 0.05$ for both targets; Figure 1). Notably, hypothalamic expression of *Ctbp2* and *Nbeal1* remained decreased after 36 h fasting followed by 6 h re-feeding with either a fat-free diet or a high-fat diet relative to control mice fed *ad libitum* (Figure 1). In line with the downregulation of hypothalamic expression of *Ctbp2* and *Nbeal1* in response to nutrient availability, expression of *Ctbp2* was increased in diet-induced obese compared with age-matched lean control mice ($P < 0.01$; Figure 2); for *Nbeal1* we noted a trend

for increased expression in obese compared with lean mice ($P = 0.070$; Figure 2).

DISCUSSION

Among the 1000 SNPs with the lowest P -values in the GCAN GWAMA for AN¹⁰ we identified nine SNPs in three chromosomal regions with significant P -values in the currently largest GWAMA for BMI variation¹³ using a conservative Bonferroni correction.

The relevance of these three loci is uncertain, because none of the nine SNPs have previously been identified for either AN or BMI/obesity or other psychiatric disorders. Two *NBEAL1* SNPs (intronic and 3'UTR, rs16839626, rs6733725; no detectable LD to the SNPs identified here; <http://www.broadinstitute.org/mpg/snap/ldsearchpw.php>) had been detected in a GWAS for obesity related traits in 815 Hispanic children from 263 families.⁴² Nominal associations (not genome-wide significant) for energy storage and fat mass deposition ($P = 2 \times 10^{-7}$), fat mass change (4×10^{-7}) and weight change (3×10^{-6}) were shown.⁴² Central (including hypothalamic) expression of all four genes was detected. We did not detect association of the previously published GWAMA SNPs for BMI, (childhood) obesity or WHR with AN (Supplementary Tables).

The following results do not readily substantiate the relevance of our association findings: (a) The analysis of the five out of nine available cross-trait SNPs in 5530 obese children and adolescents (BMI ≥ 95 th percentile) versus 8318 controls (BMI < 50 th percentile) from the EGG Consortium⁴⁰ did not reveal significant findings. However, for all available SNPs the direction of effect was identical to that observed in the GIANT GWAMA. Because the EGG Consortium GWAMA is substantially smaller than the recent GIANT approach,¹³ true signals may not have been detectable. (b) The look-up of the same cross-trait SNPs in the first GWAS for AN⁹ did not support our findings. This might partly be explained by the lower sample size in the analysis of the Price Foundation Collaborative Group and Children's Hospital of Pennsylvania samples⁹ (1033 AN cases and 3733 pediatric controls) compared with the latest GWAS¹⁰ (2907 cases with AN and 14,860 controls). In conclusion, we cannot exclude that our detected associations for the nine SNPs represent false-positive associations.

The following lines of evidence do however support that we have indeed detected SNPs associated with both AN and obesity: The identification of the three loci with nominal P -values in the range of 10^{-5} to 10^{-6} for association with BMI is quite unexpected. Accordingly, at least one and maximally all three loci are involved in body weight regulation; the same potentially holds true for AN. If this assumption is correct, future larger GWAMAs for both AN and BMI/obesity will pick up the respective loci. It is also of interest that all risk alleles were directionally consistent for AN risk and higher BMI. This is especially unexpected as (a) patients with AN do not have an elevated premorbid BMI;³³ (b) BMI-values of followed up patients only infrequently exceed the cutoff for overweight (BMI ≥ 25 kg/m²)³⁶ and (c) LD-score regression analyses revealed a negative genetic correlation between AN and obesity.³⁰ It is unlikely that the overlap between the AN (controls) and GIANT GWAMAs explains our results.

Sex-specific analyses

We also performed look-ups in sex-stratified analyses for the best cross-trait SNPs in the BMI GWAMA, because (i) AN predominantly occurs in females^{38,39} and (ii) sex-specific analyses rendered BMI loci that had not been picked up by sex-combined analyses.¹³ We found that the three AN risk SNPs at the chromosome 10 locus with the lowest P -values in the BMI GWAMA (sex-combined) mainly originated from the female participants (Table 1). This finding provides additional indirect evidence that particularly this locus is involved in both AN and body weight regulation in females mainly.

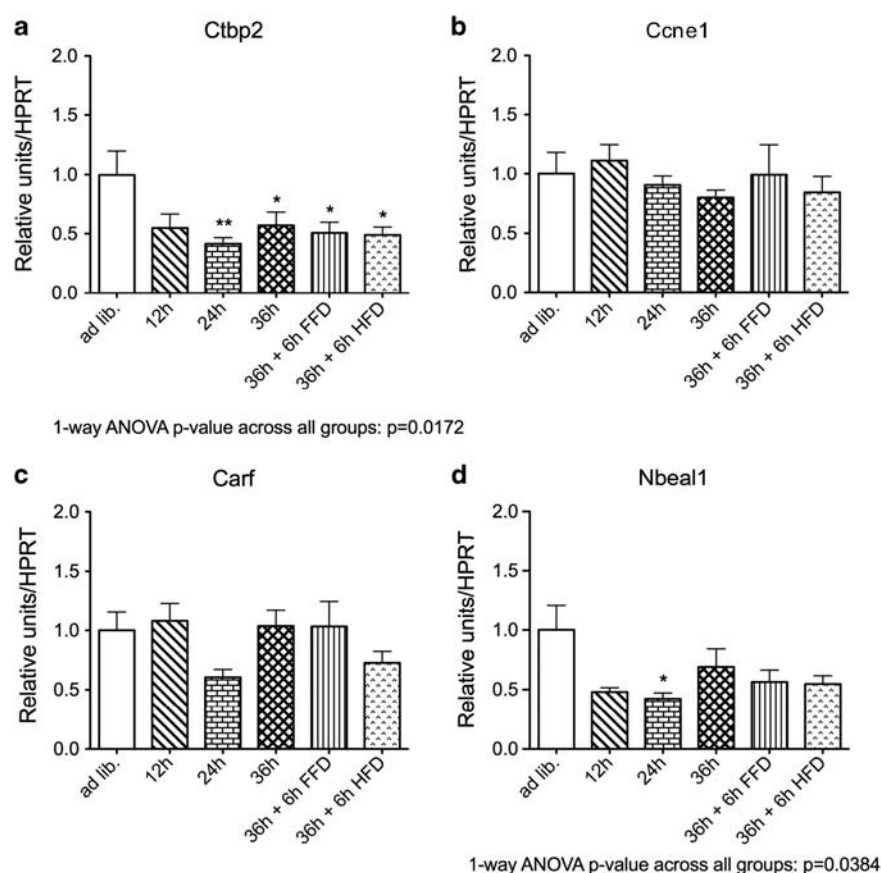


Figure 1. Hypothalamic expression of *Ctbp2* (a), *Ccne1* (b), *Carf* (c), *Nbeal1* (d), or in response to fasting for 12, 24 or 36 h, and after re-feeding for 6 h with either a high-fat diet (HFD) or a fat-free diet (FFD, $N=6-8$ mice per group) (c). * $P < 0.05$, ** $P < 0.01$, based on a one-way analysis of variance with Dunnett's multiple comparison *post hoc* test. HPRT, hypoxanthine guanine phosphoribosyltransferase 1.

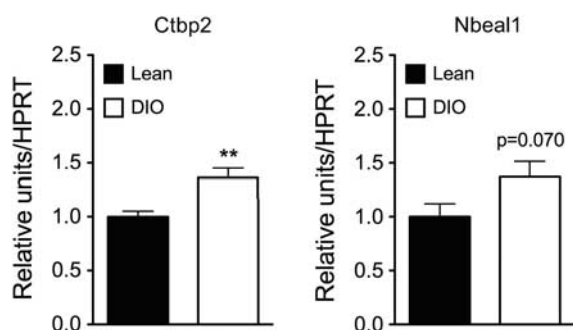


Figure 2. Hypothalamic expression of *Ctbp2* and *Nbeal1* in diet-induced obesity (DIO) as compared with age-matched lean control mice. HPRT, hypoxanthine guanine phosphoribosyltransferase 1.

Animal model

The hypothalamic expression data obtained in male mice clearly substantiate that the detected associations at two loci may indeed represent true positive findings. The cDNA of the fasting/re-feeding experiment described in this manuscript is commonly used in the Müller/Tschöp lab to assess regulation of target genes. Whereas unfortunately there is no documentation on the total number of previously analyzed targets, it can be confirmed that only few of the previously analyzed genes have been found to be differentially regulated under the conditions reported here.⁴³ Expression of *Ctbp2*, whose locus represented our strongest

association signal (Table 1), proved to be inversely regulated by fasting and diet induced obesity. Thus, hypothalamic gene expression was reduced for this gene and additionally in fasted (12, 24 or 36 h) mice; this downregulation persisted 6 h after renewed access to *ad libitum* feeding (re-feeding for 6 h with either a high-fat diet or a fat-free diet). Genes, whose expression is downregulated in fasting, are usually anorexigenic (e.g. leptin^{44,45}), while expression of orexigenic genes (e.g. ghrelin⁴⁶) is increased in fasting. Hence it is likely that both *Ctbp2* and *Nbeal1* have an anorexigenic effect. In accordance with this assumption, both genes were upregulated in DIO (Figure 2).

BDNF signaling

It is of interest to point out that the two genes *CTBP2* and *CARF* are involved in brain derived neurotrophic factor (BDNF) signaling pathways.⁴⁷⁻⁶³ The leptinergic-melanocortinergic-BDNF pathway includes genes with known genetic variation underlying both monogenic and polygenic obesity.⁶⁴ Multiple SNPs near *BDNF* are genome-wide significantly associated with obesity.^{13,65} Evidence for an involvement of BDNF in AN stems from studies on (a) animal models, (b) genetics and (c) serum or brain levels of BDNF. However, some of the data are equivocal. In more detail: (a) in animal models the central infusion of BDNF induces weight loss.^{66,67} The suppressive effects of BDNF on feeding behavior and body weight are mediated by corticotropin-releasing factor and hypothalamic neuronal histamine in mice.⁶⁸ BDNF signaling is altered by reduced BDNF expression in the hippocampus, in activity-based anorexia in mice⁶⁹ and in immobilization stress induced anorexia in rats.⁷⁰ Deletion of the *Bdnf* gene in the

paraventricular nucleus of hypothalamus resulted in hyperphagia, reduced locomotor activity, impaired thermogenesis and severe obesity. Additionally, in response to cold exposure BDNF expression in the paraventricular nucleus of hypothalamus was increased.⁷¹ (b) Association of variation in BDNF with AN was shown by some but not all studies.^{72–84} For the widely studied *BDNF* Val66Met variant, a recent meta-analysis showed no association of the infrequent 66Met allele with AN.⁸² (c) Decreased serum and brain levels of BDNF had unequivocally been reported in patients with AN.^{66,85–100} This was recently confirmed in a meta-analysis.⁹⁷ While only one study has suggested an interaction between *CTBP2* and BDNF,⁴⁷ the interaction of *CARF* and BDNF has been substantiated in numerous studies (see above). Thus, again as BDNF might be involved in both AN and obesity^{27,101,102} this gene is biologically highly plausible.

In the following we provide additional information on the genes located nearest to the three loci identified via the nine SNPs starting with the chromosome harboring the SNPs with the lowest *P*-values.

Chromosome 10

The three intronic SNPs in the *CTBP2* gene (C-terminal binding protein 2) show the lowest *P*-values in our BMI GWAMA¹³ look-up (Table 1); as stated above the effect is almost only due to females. The two alternative *CTBP2* transcripts lead to two distinct proteins, one of which is a transcriptional repressor, while the other is a major component of synaptic ribbons, a specialized form of synapses. A NAD⁺ binding domain is common to both isoforms. There is evidence that the gene/protein is involved in brown adipose tissue function and regulation.^{103–109} *Ctbp2* knock-out mice displayed abnormal phenotypes in the cardiovascular and central nervous systems, in addition to having effects on embryogenesis, growth/size/body and mortality/aging (<http://www.informatics.jax.org/allele/MGI:2183646>¹¹⁰). Recently, an miRNA that was upregulated during the development of obesity in mice (miR-342-3p) was described to promote a suppressing effect on *Ctbp2*,¹¹¹ again underscoring the relevance of the gene for weight regulation.

Chromosome 19

The cyclin E1 gene (*CCNE1*) identified via the two SNPs 5' to this gene encodes a protein that belongs to the highly conserved cyclin family. Cyclins act as (i) regulators of specific kinases and (ii) contribute to the coordination of mitotic events. In many tumors overexpression of this gene has been observed.¹¹² It was recently shown that proliferation of 3T3-L1 preadipocytes promoted by recombinant myostatin increased expression of proliferation related genes (e.g. cyclin E1 by 20.5%¹¹³).

Chromosome 2

The third chromosomal locus includes two genes, because the four SNPs are located in a region with high LD (lowest LD for the four SNPs: $r^2 \geq 0.819$, $D' = 1$). Three of the SNPs are located in an intron, one is 5' to *NBEAL1* (Table 1): (1) The calcium-response factor gene (*CARF* or as an alias name amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 8 gene: *ALS2CR8*) acts as a transcriptional activator that mediates the calcium- and neuron-selective induction of BDNF expression.⁵² Lack of *Carf* (*Als2cr8*) in knock-out mice results in deficits associated with learning and memory.⁵⁶ Functionally relevant recessive mutations in the gene have been described in patients with amyotrophic lateral sclerosis 2 (*ALS2*)¹¹⁴.

CONCLUSION

In sum, in a cross-trait analysis for genetic loci involved in AN risk and increased BMI three chromosomal loci with potential relevance for both traits were detected. Apart from the

identification of these loci, their role in both AN and body weight regulation was particularly substantiated by *ex vivo* data of mouse models for fasting and DIO, suggesting an anorexic role of *CTBP2* and *NBEAL1*, by the sex-specific results for *CTBP2* and the finding that *CTBP2* and *CARF* are involved in *BDNF* regulation. Further in-depth molecular genetic and biological analyses are essential to understand the relevance of these loci and the genes they contain in the etiology of AN and in body weight regulation/obesity. The association of AN alleles with increased BMI might imply that a specific genetic variant (allele) can either increase or decrease BMI depending on the presence or absence of additional factors with an influence of the body weight (e.g. occurrence of an eating disorder), or the variant predisposes to dysregulation and other genes or environmental factors determine its direction). If true, this general concept has implications for gene mapping approaches in genetic epidemiology calling for more hypothesis-driven stratified analyses. A spectrum of different variants (missense, nonsense and frameshift) has been described for the four genes (Supplementary Table 7), so that a mutation screen in these genes in study groups of patients with AN or extreme obesity is warranted.

CONFLICT OF INTEREST

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