Human Obesity Associated with an Intronic SNP in the Brain-Derived Neurotrophic Factor Locus

Graphical Abstract

Highlights

- BDNF rs12291063 minor C allele is associated with obesity in children and adults
- Minor C allele disrupts binding and transactivation by hnRNPD0B
- Minor C allele is associated with lower human hypothalamic BDNF expression
- BDNF augmentation could be a CC genotype-specific targeted therapy for obesity

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In Brief

Mou et al. show that brain-derived neurotrophic factor (BDNF) rs12291063 minor C allele disrupts binding and transactivation by the transcriptional regulator, heterogeneous nuclear ribonucleoprotein D0B, and it is associated with lower ventromedial hypothalamic BDNF expression and obesity. BDNF augmentation may be specifically beneficial for treating obesity in individuals with the CC genotype.

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Human Obesity Associated with an Intronic SNP in the Brain-Derived Neurotrophic Factor Locus

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SUMMARY

Brain-derived neurotrophic factor (BDNF) plays a key role in energy balance. In population studies, SNPs of the BDNF locus have been linked to obesity, but the mechanism by which these variants cause weight gain is unknown. Here, we examined human hypothalamic BDNF expression in association with 44 BDNF SNPs. We observed that the minor C allele of rs12291063 is associated with lower human ventromedial hypothalamic BDNF expression (p < 0.001) and greater adiposity in both adult and pediatric cohorts (p values < 0.05). We further demonstrated that the major T allele for rs12291063 possesses a binding capacity for the transcriptional regulator, heterogeneous nuclear ribonucleoprotein D0B, knockdown of which disrupts transactivation by the T allele. Binding and transactivation functions are both disrupted by substituting C for T. These findings provide a rationale for BDNF augmentation as a targeted treatment for obesity in individuals who have the rs12291063 CC genotype.

INTRODUCTION

Genetic factors play a role not only in the predisposition to obesity (Loos, 2012), but also in the effectiveness of obesity treatments (Choquet and Meyre, 2011). Genetic variation of the brain-derived neurotrophic factor (BDNF) locus is an important potential therapeutic target because BDNF plays a key role in energy...
homeostasis by functioning as a downstream regulator of the leptin-proopiomelanocortin pathway (Xu et al., 2003). Bdnf+/− mice (Lyons et al., 1999) and BDNF+/− humans (Han et al., 2008) exhibit hyperphagic behavior and obesity. BDNF is abundantly expressed in the ventromedial hypothalamus (VMH) (Xu et al., 2003), and selective deletion of Bdnf from the VMH and dorsomedial hypothalamus leads to obesity in mice (Unger et al., 2007). In human studies, associations have been observed between obesity and SNPs of the BDNF gene locus, most of which are intronic (Gong et al., 2013; Speliotes et al., 2010).

With the emerging evidence that non-coding genetic variants play an important role in gene regulation (Cooper, 2010), we hypothesized that SNPs within intronic regions of the BDNF locus could alter hypothalamic BDNF expression and, thereby, influence energy balance and serve as potential therapeutic targets for genotype-specific treatment of obesity. We examined the association of the BDNF locus SNPs with human VMH BDNF expression and body composition in multiple pediatric and adult cohorts. We then investigated the mechanistic role of intronic SNP rs12291063, which emerged as the strongest predictor of hypothalamic BDNF expression and body mass index (BMI).

RESULTS

The rs12291063 CC Genotype Is Associated with Decreased BDNF Expression in Human VMH

Relative expressions of the five most abundant BDNF transcripts in human hypothalamus (I, IIb, IIc, IV, and VIb) (Han et al., 2008) were measured by quantitative real-time PCR in postmortem human VMH-region tissue obtained from 84 adults (Table S1). Subjects were genotyped for 44 SNPs within or near the BDNF locus (Table S2). Of the 44 SNPs examined, only rs12291063 was significantly associated with BDNF expression after correction for multiple comparisons. Minor allele rs12291063 CC genotype was significantly associated with lower BDNF transcript IIb and nominally associated with lower BDNF transcript VIb expressions (Figure 1A). Upstream of coding exon IX, rs12291063 is located within the intron between noncoding exons VIII and VIIIh (Figure S1). Additional BDNF SNPs showing nominal associations with BDNF expression that were not significant after correction for multiple comparisons are indicated in Table S2. Because minor allele frequency (MAF) for rs12291063 is higher in African-American compared to Non-Hispanic Caucasian subjects, we confirmed the nominal associations of rs12291063 with BDNF transcripts IIb and VIb in the sub-cohort of 54 African-American subjects (p = 0.002 and p = 0.006, respectively).
The rs12291063 CC Genotype Is Associated with Greater BMI and Adiposity

**Postmortem Adult Cohort**

Subjects with the rs12291063 CC genotype had significantly greater BMI compared to subjects with the TT genotype ($p = 0.007$; Figure 1B) and a trend toward greater BMI compared to CT subjects ($p = 0.06$; Figure 1B). BMI was not significantly different between CT and TT groups ($p = 0.19$). After adjustment for age, sex, and race, the CC genotype remained significantly associated with higher BMI when compared with combined CT and TT subjects ($p = 0.03$; Figure 1C). We also confirmed the association of rs12291063 with BMI in the sub-cohort of African-American subjects ($p = 0.04$ in one-tailed analysis; data not shown).

**Adult African-American Cohorts**

Because the MAF of rs12291063 is higher in African-American compared to Caucasian cohorts (Sherry et al., 2001), we examined the association between rs12291063 and obesity in a sample of 29,151 adult subjects of African-American race who were enrolled in the Population Architecture using Genomics and Epidemiology (PAGE) consortia study (Gong et al., 2013). The rs12291063 MAF for C was 0.30 in this cohort. The number of C alleles was positively associated with BMI (adjusted for age, sex, and the first ten component vectors from multidimensional scaling to account for population substructure; $p = 0.02$, $β = +0.01$) and fat mass (additionally adjusted for height$^2$; $p = 0.04$, $β = +0.06$), and a trend toward association was observed for BMI ($p = 0.07$, $β = +0.02$).

**Healthy Pediatric Cohort**

The association between rs12291063 and obesity was further studied in a cohort of 837 healthy children (age $12.6 \pm 3.3$ years; 58% female; 53% Non-Hispanic Caucasian, 36% African-American; BMI-Z $1.24 \pm 1.18$) recruited as volunteers for clinical studies at the NIH (Table S4). The rs12291063 MAF for C was 0.02, 0.34, and 0.17 for Non-Hispanic Caucasian, African-American, and Asian/Hispanic/other subjects, respectively. Genotype distributions within racial/ethnic subgroups were consistent with Hardy-Weinberg equilibrium (HWE) ($p$ values $> 0.05$). The rs12291063 CC subjects had significantly higher BMI and higher BMI-Z than CT and TT subjects ($p$ values $< 0.01$; Figures 2A and 2B). BMI was also higher in the CT subjects compared to the TT subjects ($p = 0.049$), but BMI-Z was not significantly different ($p = 0.13$). We also confirmed the association of rs12291063 with BMI-Z in the sub-cohort of African-American children ($p = 0.04$ in one-tailed analysis; data not shown). Among 726 children who underwent body composition analysis, subjects with the rs12291063 CC genotype had a significantly greater percentage of body fat and fat mass compared to the CT and TT subjects ($p$ values $< 0.05$; Figures 2C and 2D). CT and TT subjects had similar adjusted percentage of body fat ($p = 0.84$) and similar adjusted fat mass ($p = 0.96$).
ally adjusted for height; \( p = 0.09 \), and sex; \( p = 0.04 \),
alleles was positively associated with BMI (adjusted for age
0.33. In one-tailed replication analyses, the number of minor C
Baylor College of Medicine, the rs12291063 MAF for C was
observed for BMI-Z (\( p = 0.08 \), \( 1076 \) Cell Reports
were enrolled in the Viva La Familia Study (Butte et al., 2006)a t
In a sample of 813 pediatric subjects of Hispanic ethnicity who
Replication in a Pediatric Hispanic Cohort
compared to the C allele (lane 3) probe. Table S5 shows the isolated proteins
and 40 kDa in size that appear higher in intensity for the T allele (lane 2) probe
proteins to the beads. (Lanes 2 and 3) Black bracket shows bands between 30
(Lane 1) No-oligonucleotide control showing nonspecific binding of nuclear
Figure 3. Nuclear Extract Proteins between 30 and 40 kDa Size with
Greater Binding to the Major T Allele of rs12291063
(Lane 1) No-oligonucleotide control showing nonspecific binding of nuclear
proteins to the beads. (Lanes 2 and 3) Black bracket shows bands between 30
and 40 kDa in size that appear higher in intensity for the T allele (lane 2) probe
compared to the C allele (lane 3) probe. Table S5 shows the isolated proteins
identified by mass spectrometry. (Lane 4) YY1 protein was selected as an
ubiquitous transcription factor to serve as an additional molecular weight
standard (68 kDa). (Lane 5) Molecular weight standard is shown.
Replication in a Pediatric Hispanic Cohort
In a sample of 813 pediatric subjects of Hispanic ethnicity who
were enrolled in the Viva La Familia Study (Butte et al., 2006) at
Baylor College of Medicine, the rs12291063 MAF for C was
0.33. In one-tailed replication analyses, the number of minor C
alleles was positively associated with BMI (adjusted for age
and sex; \( p = 0.04 \), \( \beta = +0.09 \)), a trend toward association was
observed for BMI-Z (\( p = 0.08 \), \( \beta = +0.07 \)) and fat mass (addition-
ally adjusted for height; \( p = 0.09 \), \( \beta = +0.07 \)), and no signif-
icient association was observed for percentage of body fat (\( p = 0.19 \)).
Transcriptional Enhancer hnRNPD0B Binds
Preferentially to Major T Allele Sequence at rs12291063
Locus
To examine the mechanism through which rs12291063 might
alter BDNF expression and lead to obesity, we used Genomatix
SNPInspector software to predict potential transcription factor
binding at the rs12291063 locus, and we found that its major T
allele sequence matches the AAATACC motif of the Mt site,
located upstream of the human DES gene. Mt binds to an
unidentified nuclear extract protein, designated as Mt-binding
factor, that is required for full transcriptional activity of DES
(Gao et al., 1998).
We hypothesized that the T allele for SNP rs12291063 is a
binding site for a transcriptional regulatory protein and that pre-
ence of the C allele decreases binding of this protein. We per-
formed a streptavidin-agarose bead pull-down procedure
(Deng et al., 2003) to isolate HeLa nuclear proteins that bind prefer-
entially to the T allele sequence of rs12291063. We observed
that protein bands between 30 and 40 kDa in size were more
intense for the T allele compared to the minor C allele (Figure 3).
Sequence by mass spectrometry of nuclear proteins that
bound to the major T allele of rs1229063 identified a total
of 23 proteins (Table S5). From this list, we selected heterogeneous
nuclear ribonucleoprotein (hnRNP)D0B (NP_002129.2;
AAB96683.1) as the most likely candidate protein because of
its molecular weight (~32 kDa), relative abundance (11.3%),
high binding affinity to DNA in a sequence-specific manner,
and known role as a transcriptional enhancer (Tolnay et al.,
To confirm that hnRNPD0B binds to the rs12291063 locus, we
conducted electrophoretic mobility shift assay (EMSA) experi-
ments. We observed that an HeLa nuclear protein bound prefer-
entially to the T allele sequence versus the C allele sequence of
rs12291063, with 50% lower binding intensity for the minor allele
sequence (\( p < 0.0001 \); Figures 4A and 4B). Competition assays
with unlabeled T allele probe showed significantly diminished in-
tensity of the labeled major T probe-protein band (\( p < 0.006 \);
Figures 4A and 4B), but no diminution from unlabeled C
allele probe (\( p > 0.11 \); Figures 4A and 4B), confirming
that the observed DNA-protein band for the T allele probe is
sequence specific and that the nuclear protein has a higher affin-
ity for the T allele over the C allele.
We proceeded to perform supershift experiments using a
mouse monoclonal pan-anti human hnRNP antibody (Figure 4C).
The primary DNA-protein band was diminished by the addition of
a pan-hnRNP antibody and a supershifted complex formed. In
contrast, mouse IgG did not alter the primary DNA-protein
band, which confirmed the specificity of the observed supershift.
A similar pattern was observed for both the T and C allele se-
quences of rs12291063, but the band intensities were lower for
the C allele, consistent with its decreased binding capability for
an hnRNP.
We then performed EMSA supershift experiments using puri-
fied recombinant hnRNPD0B protein (Figure 4D). We observed
that hnRNPD0B bound to both the T and C allele sequences,
but with lower intensity for the C allele sequence. These DNA-
protein complexes were supershifted by the addition of a
pan-hnRNP antibody, but the supershifted band intensities still
remained consistently lower for the C allele. The addition of
mouse IgG induced supershifted bands of comparatively lower
molecular weight to form, but the original DNA-protein com-
plexes were still present, suggesting that IgG nonspecifically
binds to a site on the biotin-labeled oligonucleotide distinct
from the DNA site that binds hnRNPD0B.
Minor C Allele of rs12291063 Decreases Luciferase
Reporter Gene Expression
Because hnRNPD0B has been shown to enhance transcription in
vitro (Tolnay et al., 2000), we hypothesized that substitution of T with C at rs12291063 leads to decreased binding of
hnRNPD0B, resulting in decreased gene expression. We cloned
the T or C allele and the identical 250-bp flanking sequence
of rs12291063 into pGL3-SV40 luciferase reporter vectors
Figure 5A in HEK293 cells. The construct containing the C allele exhibited 46% lower luciferase activity compared to the construct containing the T allele (p = 0.003; Figure 5B).

**Transactivation Function of the Major T Allele of rs12291063 Is Disrupted by Reduction of hnRNPD Protein Expression**

We performed small interfering RNA (siRNA)-mediated knock-down of hnRNPD0B to confirm that the observed decrease in expression was due to diminished hnRNPD0B binding at the C allele. siHNRNPD significantly decreased hnRNPD protein expression (p < 0.01) in both T and C allele construct-containing cells (Figure 5C). The siHNRNPD treatment was associated with 22% lower luciferase expression induced by the major T allele compared to siControl (p < 0.01; Figure 5D). However, there was no significant difference in luciferase expression between siHNRNPD and siControl in cells containing the minor C allele (p = 0.2; Figure 5D), thus supporting a specific transactivation activity induced by binding of hnRNPD0B with the major T allele of rs12291063. Furthermore, cells co-transfected with the minor C allele and siControl showed a significant decrease in luciferase activity compared to cells co-transfected with the major T allele and siControl (p = 0.000005; Figure 5D), which serves as secondary confirmation that the minor C allele causes decreased gene expression.

**DISCUSSION**

We observed that the rs12291063 CC genotype was associated with lower expressions of BDNF transcripts IIb and VIb in human VMH and higher BMI and adiposity in multiple racially diverse pediatric and adult cohorts. Our in vivo and clinical findings indicate...
average of 29% lower HnRNPD protein expression compared to matched cells with siControls (n = 4; p = 0.0008). Cells containing the T allele construct and siHnRNPD exhibited an average of 33% lower HnRNPD protein expression compared to matched cells with siControl (n = 8; p = 0.000005). Two-tailed, independent sample t tests were performed and means ± SEMs are shown.

that BDNF rs12291063, which had an MAF of ~30% within our Hispanic and African-American cohorts, could play an important role in the pathogenesis of obesity in these populations. Mechanistically, our data suggest that diminished binding of hnrnpd0b at the rs12291063 locus in individuals with the CC genotype could cause decreased BDNF mRNA expression and protein concentrations within the hypothalamus, potentially leading to excessive energy intake and weight gain.

Regulation of BDNF expression is complex, with multiple promoters responding to a variety of control mechanisms, giving rise to distinctly regulated mRNA variants with different brain region expression patterns (Pruunsild et al., 2007; Zheng et al., 2012). BDNF transcripts IIb and VIb comprise 38% of the total BDNF mRNA expression in the hypothalamus (Han et al., 2013). Thus, decreased expression of these two transcripts could have significant impact on body weight regulation.

Our findings provide a clinically relevant potential mechanism for the obesity risk associated with a common noncoding variant of BDNF. Genetic association studies have identified many genetic variants associated with obesity, but their clinical utility for obesity treatment is currently limited (Loos, 2012). Elucidation of the functional consequences of these genetic variants are needed to advance personalized remedies targeting specific deficits on an individual basis (El-Sayed Moustafa and Froguel, 2012). With this goal in mind, our observations could serve as the basis for developing therapies aimed at potentiating hypothalamic BDNF signaling as a specific treatment for obesity in individuals who have the rs12291063 CC genotype.

EXPERIMENTAL PROCEDURES

Please see the Supplemental Experimental Procedures for additional details.

Human Brain Sample Subjects

Brain tissue was obtained from the Offices of the Chief Medical Examiner of Washington, DC, and of Northern Virginia (Clinical Brain Disorders Branch, IRP, NIMH collection) at autopsy from non-neurologic non-psychiatric control subjects who suffered sudden deaths. All the tissues were donated with informed consent from the next of kin. Further details on cohort selection have been reported previously (Lipska et al., 2008). The region of VMH was dissected from frozen brain specimens using anatomic landmarks. The qPCR and SNP genotyping were performed (see the Supplemental Experimental Procedures).

Human Subjects

The following four cohorts were investigated: (1) adults of African-American race from the PAGE consortia (Gong et al., 2013; Table S3); (2) adults of African-American race from the HANDLS study (Evans et al., 2010); (3) pediatric volunteers participating in clinical studies conducted at the NIH; and (4) children and adolescents of Hispanic heritage from the Viva La Familia longitudinal study (Butte et al., 2009). Informed consent and assent were obtained from adult subjects and parents/guardians of minors. All methods for obtaining clinical data from the subjects were approved by the respective Institutional Review Board at each site.

Bioinformatics

SNPInspector software (Genomatix Software) was used to identify candidate transcription factors at the SNP loci.

Protein Isolation and Identification

A streptavidin-agarose bead pull-down procedure, as previously described (Deng et al., 2003), was used to isolate nuclear proteins that bind at the
rs12291063 locus. Excised gel bands were submitted to Protech for protein isolation and sequencing by mass spectrometry.

EMSA

The 5’-biotinylated and unabeled 25-bp oligonucleotides containing the major and minor allele sequences at the rs12291063 locus were obtained from Invitrogen. Recombinant hnRNPD0B protein expressed in E.coli was obtained from OriGene Technologies. EMSSs with competition and supershift assays were performed using LightShift Chemiluminescent EMSA kit (Pierce Protein Biology Products) and results were digitized and quantified as previously described (Schneider et al., 2012).

 Luciferase Reporter and siRNA Knockdown Assays

The 250-bp sequences containing either the major or the minor allele of rs12291063 were cloned into pGL3 luciferase reporter vector driven by an SV40 promoter (Promega) at the HindIII site between the promoter and the firefly Luc gene. HEK293 cells were cultured and transiently transfected with the constructs using FuGENE HD Transfection Reagent (Promega). For luciferase assay activity with hnRNPD depletion, HEK293 cells were transiently co-transfected with hNRV4 siRNA or Control siRNA (SignalSilence, Cell Signaling Technology), Dual Luciferase Reporter Assay System (Promega) was used to quantify luciferase activity. Efficiency of siRNA knockdown for hnRNPD was verified by western blot using pan-hnRNPD antibody and GAPDH antibody (Santa Cruz Biotechnology).

Statistical Analyses

SPSS Statistics Version 17.0 software (IBM) was used for statistical analyses. Univariate analyses of covariance (ANCOVAs) with post hoc pairwise least significant difference (LSD) comparisons were performed to assess the association of genotype with BDNF expression, BMI, BMI-Z, body fat percentage, and fat mass. EMSA band intensities from three separate experiments were compared using ANOVA with post hoc LSD. Firefly luciferase activity and hnRNPD protein expression were compared using two-tailed, independent sample t tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one figure, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.065.

AUTHOR CONTRIBUTIONS


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