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ORIGINAL RESEARCH ARTICLE

Candidate genes for anorexia nervosa in the 1p33–36 linkage region: serotonin 1D and delta opioid receptor loci exhibit significant association to anorexia nervosa

AW Bergen[1,13,16], MBM van den Bree[1,14,16], M Yeager[1,15], R Welch[1,15], JK Ganjei[1], K Haque[1,15], S Bacanu[2], WH Berrettini[3], DE Grice[3], D Goldman[4], CM Bulik[5], K Klump[6], M Fichter[7], K Halmi[8], A Kaplan[9], M Strober[10], J Treasure[11], B Woodside[9] and WH Kaye[12]

[1]*Biognosis US, Inc. (Dissolved). From the Price Foundation Collaborative Group;* [2]*University of Pittsburgh School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA;* [3]*Center for Neurobiology and Behavior, Department of Psychiatry, University of Pennsylvania School of Medicine, Philadelphia, PA, USA;* [4]*National Institute of Alcohol Abuse and Alcoholism, Rockville, MD, USA;* [5]*Department of Psychiatry, Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, USA;* [6]*Department of Psychology, Michigan State University, East Lansing, MI, USA;* [7]*Klinik Roseneck, Hospital for Behavioral Medicine, Munich, Germany;* [8]*New York Presbyterian Hospital-Westchester, Weill Medical College of Cornell University, White Plains, New York, USA;* [9]*Department of Psychiatry, The Toronto Hospital, Toronto, Canada;* [10]*Neuropsychiatric Institute and Hospital, School of Medicine, University of California at Los Angeles, Los Angeles, USA;* [11]*Institute of Psychiatry, Maudsley and Bethlehem Royal Hospital, London, UK;* [12]*Eating Disorders Module, Western Psychiatric Institute & Clinic, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*

Serotonergic and opioidergic neurotransmitter system alterations have been observed in people with eating disorders; the genes for the serotonin 1D receptor (HTR1D) and the opioid delta receptor (OPRD1) are found on chr1p36.3–34.3, a region identified by our group in a linkage analysis of anorexia nervosa (AN). These candidate genes were evaluated for sequence variation and for linkage and association of this sequence variation to AN in family and case : control data sets. Resequencing of the HTR1D locus and a portion of the OPRD1 locus identified novel SNPs and confirmed existing SNPs. Genotype assay development and genotyping of nine SNPs (four at HTR1D and five at OPRD1) was performed on 191 unrelated individuals fulfilling DSM-IV criteria (w/o amenorrhea criterion) for AN, 442 relatives of AN probands and 98 psychiatrically screened controls. Linkage analysis of these candidate gene SNPs with 33 microsatellite markers in families including relative pairs concordantly affected with restricting AN (*N*=37) substantially increased the evidence for linkage of this region to restricting AN to an NPL score of 3.91. Statistically significant genotypic, allelic, and haplotypic association to AN in the case : control design was observed at HTR1D and OPRD1 with effect sizes for individual SNPs of 2.63 (95% CI=1.21–5.75) for HTR1D and 1.61 (95% CI=1.11–2.44) for OPRD1. Using genotype data on parents and AN probands, three SNPs at HTR1D were found to exhibit significant transmission disequilibrium ($P<0.05$). The combined statistical genetic evidence suggests that HTR1D and OPRD1 or linked genes may be involved in the etiology of AN.
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Correspondence: WH Kaye, MD, Department of Psychiatry, 3811 O'Hara St, 600 Iroquois Building, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA.
E-mail: kayewh@msx.upmc.edu
[13]Current address: Core Genotyping Facility, Advanced Technology Center, National Cancer Institute, Gaithersburg, MD 20877, USA.
[14]Current address: Division of Psychological Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK.
[15]Current address: Core Genotyping Facility, Advanced Technology Center, National Cancer Institute/SAIC, Gaithersburg, MD 20877, USA.
[16]These two authors contributed equally.
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Introduction

Anorexia nervosa (AN) is a disorder predominantly affecting women, and most often has its onset during adolescence. It is characterized by pathologic eating behavior and the relentless pursuit of thinness, resulting in extreme emaciation. As a result of its physical morbidity and potential refractoriness to treatment, AN has the highest mortality among the psychiatric disorders.[1] Depression, anxiety, and obsessive–compulsive behaviors are common psychopathological features in AN. Moreover, people with AN tend to have a unique cluster of personality and temperamental traits including perfectionism, over control, rigidity, and harm avoidance. Such behaviors may constitute predisposing traits since they occur

premorbidly and frequently persist well after weight and eating normalize.[2–4] Recent studies show that AN is familial,[5,6] with heritability estimates derived from large-scale community-based twin studies ranging between 0.5 and 0.8.[7–9]

Human and animal studies raise the possibility that alterations within a variety of neuromodulating systems may contribute to aspects of the appetitive and behavioral abnormalities in AN, including the serotonin[10,11] and opioid[12] neurotransmitter systems. Alterations in serotonin activity have been implicated in disturbances of mood and impulse control.[13–15] In fact, most studies have shown that individuals with AN have disturbances of central serotonin activity,[15–17] which persist after recovery.[18–20] The opioid system has been implicated in the modulation of feeding behavior in which opioid agonists and antagonists were injected into animals.[21–24] Some clinical studies have suggested that opioid antagonists may have beneficial effects on eating behavior symptoms in patients with eating disorders.[25,26]

We have recently completed a genomewide linkage analysis of 192 affected relative pairs with AN and related eating disorders, including bulimia nervosa (BN), which gave only modest evidence for linkage when all DSM-IV eating disorder diagnoses were included in the affected sample.[27,28] Linkage analysis in a subset of 37 families with at least one affected relative pair concordant for a DSM-IV diagnosis of AN, restricting subtype (AN1), showed suggestive evidence for linkage with the highest multipoint NPL score of 3.45 occurring on chromosome 1p33–36 at marker D1S3721, located at ~72.6 cM on the human linkage map and ~48.1 Mbp on the human genome draft sequence.[28]

The serotonin 1D (HTR1D), delta opioid (OPRD1), and hypocretin receptor 1 (HCRTR1) receptor loci reside on chromosome 1p33–36, at ~49.0 cM/25.7 Mbp, ~55.7 cM/33.5 Mbp, and 61.65 cM/36.9 Mbp, respectively. Cloning, evaluation of pharmacological properties, functional expression and coupling to G proteins, and the chromosomal location of HTR1D,[29–31] OPRD1,[32–35] and HCRTR1[36] have been previously reported. HTR1D contains a single exon of 1134 base pairs (bp) of cDNA sequence, OPRD1 contains three exons of 51 551 bp with 1173 bp of cDNA sequence, and HCRTR1 contains seven exons of 8074 bp with 1564 bp of cDNA sequence. Their protein products are cell surface receptors with ligands serotonin, met- and leu-enkephalin, and hypocretin-1, respectively. SNPs in the coding region of HTR1D,[37] OPRD1,[38,39] and HCRTR1[40] have previously been identified. There are no published association studies of HTR1D, OPRD1, or HCRTR1 sequence variation in eating disorders.

The purpose of this study was (1) to identify novel sequence variation and to confirm previously reported sequence variation at HTR1D, OPRD1, and HCRTR1, (2) to genotype this sequence variation in individuals fulfilling DSM-IV criteria for AN and in psychiatrically and obesity screened controls, (3) to genotype additional probands, affected and unaffected relatives if nominally significant association was observed in case : control contingency analysis of AN phenotype, and (4) to evaluate association and linkage between sequence variants at these candidate genes and DSM-IV AN.

Materials and methods

Sample ascertainment

A multicenter collaborative study on the genetics of AN and BN supported by the Price Foundation has collected a large sample of eating disorder affected relative pairs ($N=220$) ascertained on an anorexic nervosa proband (www.anbn.org).[27] DNA from that study was available from 196 probands, from 182 parents of probands and from 260 affected relatives, including 52 affected parents. The probands in this sample are 95% female with a minimum past body mass index (BMI) of $14.27 \pm 2.88\,kg/m^2$. The geographical ascertainment of the family sample was from North America (70%), the UK (11%), and Germany (19%). A European-American female ('EAF') sample with $N=98$ DNA samples available was used as a control sample for genotype, allele, and haplotype frequency comparisons. The EAF sample was recruited through advertisements and screened to exclude obese individuals (>20% ideal weight) and lifetime criteria for Axis I disorders as assessed by clinical administration of the Structured Clinical Interview for DSM-III-R.[41] Informed consent was obtained from all AN study participants and all sites received approval from their local institutional review board as described.[27] The EAF control sample was interviewed and provided a blood sample as part of an IRB-approved clinical collection. Biognosis US, Inc. investigated biomaterials and clinical phenotypes under the terms of a Laboratory Sciences and Development Agreement. Unrelated Centre Étude Polymorphism Humaine (CEPH) DNA samples obtained from Coriell Cell Repositories after stipulating that the DNA samples were to be used for research use only were used for resequencing, genotype assay development, and as individual control DNAs for genotype assays.

Identification of candidate genes and polymorphisms

Identification of candidate genes, genomic sequence, and sequence variation was accomplished using public sequence and variation databases and from a genomic sequence determined from unrelated CEPH DNA samples at Biognosis US, Inc. Genomic sequence locations reported here are from the draft human genome sequence, August 6, 2001 assembly (www.genome.ucsc.edu), and linkage map locations reported here are from the genetic map of eight CEPH families produced by the Marshfield Medical Foundation (http://research.marshfieldclinic.org/genetics/Default.htm). Alignment and annotation of cDNA and genomic sequence at candidate genes used the

Sequencher™ sequence evaluation package version 4.0.5 (Gene Codes Corporation, Ann Arbor, MI, USA).

Resequencing of HTR1D, OPRD1, and HCRTR1 to identify novel SNPs

Except as noted, 32 unrelated CEPH DNAs were used in resequencing candidate genes to conserve AN proband DNA, which was available only from a limited amount of blood and not from cell lines. The HTR1D locus (from –1165 bp 5′ to +1497 bp) was resequenced to validate previously reported HTR1D SNPs and to identify novel HTR1D sequence variation using the oligonucleotide primers for PCR and cycle-sequencing listed in Table 1 of the web supplement. A 4.18 kb portion of the genomic sequence surrounding exon 2 of OPRD1 (from IVS1−1860 to IVS2+1996) was resequenced to identify additional OPRD1 sequence variation using the oligonucleotide primers for both PCR and cycle-sequencing listed in Table 1 of the web supplement. The locus HCRTR1 (from −1130 bp 5′ to 1003 bp 3′ of the termination codon) was resequenced in 32 unrelated CEPH DNAs and (the exons only) in 29 AN proband DNAs. The general PCR conditions for resequencing were (per 50 μl reaction): 50 ng genomic DNA, 25 nM each of the forward and reverse primers, 10 mM dNTP, 50 mM $MgCl_2$, 160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween-20, and 2.5 U *Taq* DNA polymerase (Bioline, Springfield, NJ, USA). General conditions for the PCR were: 94°C for 1 min, followed by 30 cycles of 94°C for 15 s, T_a°C for 30 s (where T_a°C was 65°C for all primer pairs with the following exceptions: 62°C for PF-0075/PR-0074, PF-0010/PR-0011 and PF-0087/PR-0088 and 55°C for PF-0078/PR-0079), 72°C for 1 min, with a final extension step of 72°C for 5 min. Post-PCR, the 50 μl PCR reaction was purified by Millipore PCR Purification System (Millipore Corporation, Danvers, MA, USA). Products were then quantitated and 0.25 μg of purified PCR product was cycle-sequenced with 25 pM primer, 4 μl Big Dye Terminators (PE Biosystems, Foster City, CA, USA) in a 20 μl reaction as per the manufacturer's directions.

Genotyping

For each gene, multiple SNPs were selected for genotyping using the 5′ exonuclease assay using the criteria of location, allele frequency, and polymorphism effect. Probes and primers were chosen using ProbeITY (Celadon Laboratories, College Park, MD, USA) and were synthesized by Applied Biosystems (Foster City, CA, USA). General conditions for end point-read TaqMan™ PCR were as described,[42] except for OPRD1 (47821A>G), where 300 nM of each of the two probes, and for HTR1D(−1123T>C), where 300 nM of the TET probe was used. See Table 2 of the web supplement for primer and probe sequences used in these TaqMan genotyping assays. Genotype determination was conducted manually by a technician using Applied Biosystems software on the Applied Biosystems Sequence Detector 7700 (Applied Biosystems, Foster City, CA, USA). For each assay, 653 AN probands, affected siblings, and other family members were genotyped, as well as an additional 244 control samples from different sources. A verification plate consisting of 17% of the AN probands and control group samples was genotyped in order to assess the reproducibility of the assay. Quality control procedures in the laboratory included genotyping of a duplicated sample ($N=72$) to assess genotyping error rate, no template (no genomic DNA) controls for genotype assay quality control, and Hardy–Weinberg equilibrium (HWE) tests for overall genotype error checking. Observed discordant genotypes were dropped from analysis.

Statistical analyses

HWE was evaluated using contingency analysis. Multilocus HTR1D and OPRD1 genotypes were assembled in a Visual Basic utility and resulting multilocus genotype counts were used to estimate intragenic and intergenic pairwise linkage disequilibrium using likelihood ratio tests with empirical significance testing (using 10 000–16 000 permutations) using Arlequin.[43] To evaluate whether the candidate genes HTR1D and OPRD1 might be linked to the chr1p region previously observed to be linked to restricting AN subtype AN1,[28] linkage analysis was performed using genotypes from HTR1D and OPRD1 SNPs and 33 chr1 microsatellite markers from the linkage scan, restricted to those families ($N=37$) with at least one relative-pair concordantly affected with the restricting anorexia subtype (AN1) using GENEHUNTER in a nonparametric allele-sharing approach.[44] To place HTR1D and OPRD1 on the genetic map, their locations were imputed by linear interpolation from the estimated locations of genes and STRs on the draft human genome sequence and the estimated locations of the STRs on the Marshfield genetic map.

The significance of differences in genotype and allele frequencies at the HTR1D, OPRD1, and HCRTR1 loci between the AN and control samples was evaluated using χ^2 analysis in SAS.[45] In case of expected cell frequency ≤ 5, the P-value was based on the Fisher exact test. The empirical significance of haplotype frequency differences between AN and control samples was evaluated using the nonparametric heterogeneity statistic (T5) in the program EH+ using 10 000 permutations.[46] In order to maximize the power to detect transmission disequilibrium at individual SNPs, FBAT was used for transmission disequilibrium analysis test (TDT).[47] To evaluate the possible relationship between HTR1D and OPRD1 linkage and association to AN, the average number of '2' alleles for all siblings for each candidate gene SNP and family was calculated arithmetically (allele frequencies were estimated by counting alleles, ignoring family relationships where most affected relative-pairs were sib-pairs), and identity-by-decent (IBD) allele sharing for siblings at each SNP for each family was estimated by GENEHUNTER.[44] These two variables were then

correlated, after Fisher's Z transformation, to evaluate the relation between linkage and association. Power and the required sample size were evaluated using χ^2 distribution properties and, for the TDT, the pedigree structures. A *P*-value of <0.05 is described in all analyses as indicating a statistically significant result, while *P*-values $\geqslant 0.05$ and <0.10 are described in all analyses to indicate a trend towards statistical significance.

Results

Identification of neurobiological candidate genes in the linkage region

Results from a linkage analysis restricting AN exhibiting suggestive evidence for linkage on chromosome 1p (NPL = 3.5 at D1S3721, 72.6 cM, 48.1 Mbp)[27] prompted a search for genes in this region with neurobiological functions that might serve as candidate genes for analysis. At the time these candidate gene studies were initiated (early 2000), sequence information from the Human Genome Project did not permit accurate *in silico* localization of genes to each other or to the human linkage map. Nevertheless, evaluation of public sequence databases (below) identified three genes thought to be relevant candidates owing to their neurobiological function: HTR1D, OPRD1, and HCRTR1, currently located at ~25.7, ~33.5, and ~36.9 Mbp from 1pter, respectively. HTR1D and OPRD1 loci were identified as positional neurobiological candidates through a search of unfinished BAC sequences from the high-throughput genome sequencing section of Genbank from BACs and BAC ends identified through BLAST[48] searches of the sequence of chr1p microsatellite markers used to identify the linked region on chr1p. HCRTR1 was identified as a positional neurobiological candidate through the biomedical literature.[36]

Genotyping

The resequencing of CEPH samples at HTR1D, OPRD1 and HCRTR1 identified five HTR1D SNPs (four SNPs *de novo* and one SNP was previously reported[37]), one OPRD1 SNP (*de novo*), and 13 HCRTR1 SNPs (*de novo*).

Four HTR1D SNPs, five OPRD1 SNPs, and four HCRTR1 SNPs were selected for genotype assay development and genotyped in the AN proband and EAF samples (see Tables 1 and 2). Average genotype completion rates were 96, 95, and 94% for the AN proband sample and 90, 87, and 90% for the EAF control sample for HTR1D, OPRD1 and HCRTR1 SNP genotyping assays, respectively. Observed discordant genotyping rates for HTR1D SNPs were 2.9, 0, 1.4, and 2.9%, for OPRD1 SNPs were 0, 0, 1.4, 1.4, and 4.2%, and for HCRTR1 SNPs were 0, 0.5, 0.7, and 0%, respectively. See Table 3 of the web supplement for absolute and relative allele and genotype frequencies of the HTR1D, OPRD1, and HCRTR1 SNPs in the AN proband and EAF control samples. The four SNPs at HCRTR1 were not evaluated in family samples or via haplotype analysis in this evaluation of chr1p36.3–34.3 linkage region candidate genes because none of the four HCRTR1 SNPs exhibited statistically significant case : control association (genotypic or allelic) or a trend towards association in contingency analyses (see below).

SNPwise equilibrium and pairwise linkage disequilibrium

HWE equilibrium (26 tests of HWE performed) was observed for all HTR1D, OPRD1, and HCRTR1 SNPs in the AN proband and EAF control samples (data not shown). A trend towards deviation from HWE was observed at OPRD1(80T>G) and HCRTR1(846A>G) in the AN proband sample only. Significant pairwise linkage disequilibrium was observed in both the AN

Table 1 HTR1D, OPRD1, and HCRTR1 SNPs genotyped

SNP[a]	*Allele 1*	*Allele 2*	*SNP ID*[b]	*Coding region*	*Source*	*% A1*[c]
HTR1D(−1123T>C)	T	C	SNP000083015	No, 5′ of coding	This study	31.3
HTR1D(−628T>C)	T	C	SNP000083091	No, 5′ of coding	This study	14.1
HTR1D(1080C>T)	C	T	SNP000006432	Yes, silent	Reference 40	9.4
HTR1D(2190A>G)	A	G	SNP000080270	No, 3′ of coding	This study	65.6
OPRD1(80T>G)	T	G	SNP000063484	Yes, F27C	Reference 42	
OPRD1(8214T>C)	T	C	SNP001026473	No, IVS 1	TSC0110129	
OPRD1(23340G>A)	G	A	SNP000085600	No, IVS 1	TSC0110127	
OPRD1(47821A>G)	A	G	SNP000066643	No, IVS 2	This Study	41.1
OPRD1(51502A>T)	A	T	SNP000066640	No, 3′ of coding	TSC0110133	
HCRTR1(114C>T)	C	T	SNP000779462	Yes, silent	This study	38.7
HCRTR1(846A>G)	A	G	SNP001026448	No, IVS 2	This study	59.4
HCRTR1(7757A>G)	A	G	SNP001026446	Yes, silent	This study	35.5
HCRTR1(8793C>T)	C	T	SNP001026450	No, 3′ of coding	This study	57.8

[a]From the ATG of the candidate gene genomic sequence.
[b]HGVBASE at http://hgvbase.cgb.ki.se/.
[c]Percent allele 1 from 32 unrelated CEPH DNAs obtained by resequencing.

Table 2 Results of case : control association analyses, AN *vs* EAF

SNP	*N*	χ^2	*P*	*OR*	*95% CI*
HTR1D(−1123T>C)					
Genotypes	272	2.59	0.27		
Alleles	544	2.46	0.12	0.73	0.50–1.08
HTR1D(-628T>C)					
Genotypes	273	4.62	0.10*		
Alleles	546	1.77	0.18	0.72	0.44–1.17
HTR1D(1080C>T)					
Genotypes	269	7.92	0.01*		
Alleles	538	6.32	0.01	2.63	1.21–5.75
HTR1D(2190A>G)					
Genotypes	273	2.97	0.23		
Alleles	546	2.64	0.10	1.37	0.94–1.99
OPRD1(80T>G)					
Genotypes	262	3.65*	0.17		
Alleles	524	0.01	0.94	0.98	0.55–1.76
OPRD1(8214T>C)					
Genotypes	261	3.87	0.14		
Alleles	522	4.01	0.045	1.46	1.01–2.13
OPRD1(23340A>G)					
Genotypes	270	3.84	0.15		
Alleles	540	4.00	0.046	0.68	0.47–0.99
OPRD1(47821A>G)					
Genotypes	258	7.05	0.03		
Alleles	516	6.32	0.01	0.61	0.41–0.90
OPRD1(51502A>T)					
Genotypes	268	4.14	0.13		
Alleles	536	3.51	0.06	0.70	0.48–1.02

*Indicates that the genotypic χ^2 analysis required the use of Fisher's exact test due to low cell counts.

proband and EAF samples among HTR1D and OPRD1 SNPs (AN proband sample linkage disequilibrium (LD) shown in Table 4 of the Web supplement, EAF data not shown). HTR1D intragenic pairwise LD among all HTR1D SNP pairs in both the AN and EAF samples was complete, that is, only three of four expected haplotypes at each HTR1D SNP pair was observed. Significant intragenic LD among OPRD1 SNP pairs was observed at eight of 10 OPRD1 SNP pairs in both AN proband and EAF samples, where three of 10 OPRD1 SNP pairs were observed to be highly significantly associated ($P<10^{-5}$) in the AN proband sample. The OPRD1(8214T>C)/OPRD1(23340A>G) SNP pair was in complete LD in both the AN and EAF samples (EAF data not shown). Significant LD was observed at two of 20 intergenic HTR1D/OPRD1 SNP pairs in the AN sample (see Table 4 of the web supplement).

Table 3 OPRD1 and HTR1D haplotype frequency heterogeneity analyses, AN *vs* EAF

SNP haplotype	χ^2	*P**
HTR1D		
(−628T>C)/(1080C>T)	8.90	0.01
(−1123T>C)/(1080C>T)	6.26	0.04
(1080C>T)/(2190A>G)	7.14	0.03
(−1123T>C)/(-628T>C)	3.34	0.19
(−628T>C)/(2190A>G)	3.28	0.22
(−1123T>C)/(2190A>G)	2.38	0.31
(−628T>C)/(−1123T>C)/ (1080C>T)/(2190A>G)	7.52	0.16
OPRD1		
(8214T>C)/(23340A>G)	4.24	0.12
(8214T>C)/(51502A>T)	6.04	0.13
(80T>G)/ (8214T>C)	5.00	0.22
(8214T>C)/(47821A>G)	8.82	0.04
(23340A>G)/(51502A>T)	5.54	0.16
(80T>G)/(23340A>G)	3.88	0.32
(23340A>G)/(47821A>G)	7.94	0.06
(80T>G)/(51502A>T)	2.86	0.43
(47821A>G)/(51502A>T)	8.90	0.06
(80A>G)/(47821T>G)	5.34	0.16
(80A>G)/(8214T>C)/(23340A>G)/ (47821T>G)/(51502A>T)	25.14	0.05

*Based on 10 000 permutations.

Linkage analysis of candidate gene SNPs and STR markers

The nonparametric linkage analysis of chromosome 1 markers produced a maximum NPL score of 3.91 at 72.0 cM ($P=4.6\times10^{-5}$), and NPL scores of 2.0 and 3.05 at the estimated linkage map locations of HTR1D (49.0 cM) and OPRD1 (55.7 cM), respectively (Figure 1). There was a significant correlation between IBD allele sharing and '2' alleles at HTR1D (1080C>T) ($r=-0.366$, $P=0.036$) and a trend towards a significant correlation for OPRD1(80T>G) ($r=-0.305$, $P=0.084$). There were no other significant or trend associations between IBD allele sharing and '2' alleles at the other seven HTR1D and OPRD1 SNPs evaluated in this manner.

Association of HTR1D, OPRD1, and HCRTR1 SNPs to DSM-IV AN

Statistically significant association of HTR1D and OPRD1 SNPs to AN phenotype was observed at one HTR1D SNP, HTR1D(1080C>T), both genotypic and allelic, and at three of five OPRD1 SNPs, OPRD1(8214T>C), allelic, OPRD1(23340A>G), allelic, and OPRD1(47821A>G), both genotypic and allelic (Table 2). A trend towards significant association was observed at two HTR1D SNPs, HTR1D(2190A>G), allelic and HTR1D(-628T>C), genotypic and at one OPRD1 SNP, OPRD1(51502A>T), genotypic. Note that removal of the males ($N=10$) in the AN proband sample, which results in entirely female case and control samples,

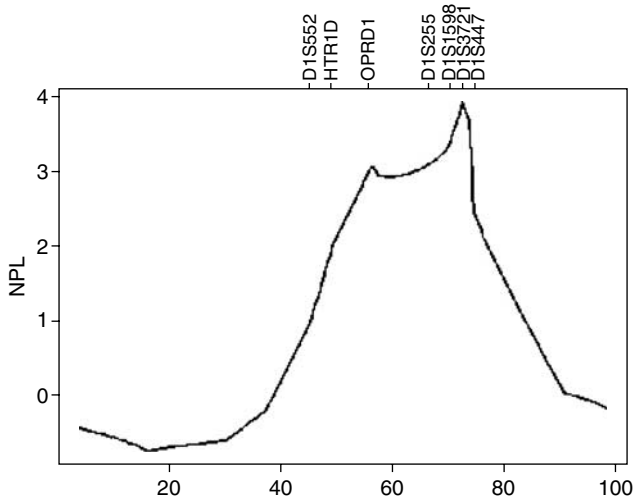

Figure 1 Distal 100 cM of chr1p with microsatellite markers and candidate genes identified. Peak is at D1S3721, 72 cM, NPL = 3.91.

increases the significance of all statistical tests by up to a factor of two, with a small increase in the risk effect of individual alleles (data not shown).

Significant HTR1D SNP haplotype frequency heterogeneity (Table 3) between the AN proband and EAF samples is observed with haplotypes containing the HTR1D(1080C > T) SNP, but not with the HTR1D haplotype containing all four SNPs. Significant OPRD1 SNP haplotype frequency heterogeneity between the AN proband and EAF samples is observed with OPRD1 SNP haplotype (8214T > C)/(47821A > G) and with the SNP haplotype containing all five SNPs. A trend towards significant haplotype frequency heterogeneity is observed with the remaining OPRD1 SNP haplotypes containing the 47821A > G SNP.

The same unrelated probands used in the case: control analyses described above were used as affected children for transmission disequilibrium analysis (Table 4). The average number of parental DNAs available for molecular genetic analysis is less than two parents per proband, limiting the number of trios available for analysis. Nevertheless, we observed significant transmission disequilibrium at three HTR1D SNPs, and a trend towards significant transmission disquilibrium at two OPRD1 SNPs.

Discussion

Intragenic and intergenic LD at OPRD1 and HTR1D

In this study, statistically significant intragenic linkage disequilibrium is observed across the entire HTR1D locus, where the HTR1D SNPs evaluated here span ~3.3 kbp. The strongest evidence of intragenic LD at the OPRD1 locus is found among one pair of SNPs located in IVS1 separated by >15 kbp, one pair of SNPs in exon 1 and 3′ of the coding region separated by 51 kbp, and a pair of SNPs in IVS1 and IVS2 separated by 39 kbp (see Table 4 of the web supplement). Nominally significant levels of OPRD1 intragenic LD are seen between an additional five SNP pairs and two SNP pairs exhibit no evidence for LD. Thus, although the OPRD1 gene is larger than the HTR1D gene, there is very significant LD among SNP pairs separated by as much as 51 kbp. The distance between the 20 HTR1D and OPRD1 intergenic SNP pairs is 7796 kbp (±21 kbp). Two of 20 intergenic observations are significant at the $P<0.05$ level, two at the $P<0.10$ level and five at the $P<0.25$ level in the AN sample (see Table 4 of the web supplement). Therefore, it appears that the number of intergenic HTR1D and OPRD1 SNP pairs observed to be in LD is equal to the number expected by chance, that is, HTR1D and OPRD1 do not appear to be in significant LD in the AN sample.

Table 4 HTR1D and OPRD1 SNP TDT results

SNP	*Freq (Allele1)*	*No. of informative families*	*Z*	*P*
HTR1D(−1123T>C)	0.281	32	−2.34	0.02
HTR1D(−628T>C)	0.150	23	−2.50	0.01
HTR1D(1080C>T)	0.112	22	1.00	0.32
HTR1D(2190A>G)	0.701	34	2.03	0.04
OPRD1(80T>G)	0.887	22	0.82	0.41
OPRD1(8214T>C)	0.558	39	0.14	0.89
OPRD1(23340A>G)	0.327	40	−0.57	0.57
OPRD1(47821A>G)	0.608	46	−1.86	0.06
OPRD1(51502A>T)	0.615	45	−1.89	0.06

Z=normalized z score of transmission statistic with *P*=two-sided *P*-value.

Linkage analysis of the chr1p region with candidate gene SNPs

Using the same sample of $N=37$ families with concordantly affected sib-pairs with restricting AN,[28] the approach of Horikawa *et al*[49] was followed by analyzing the relation between the linkage signal on chr1p and the SNP genotypes at HTR1D and OPRD1. The strength and significance of the linkage signal previously observed[28] was increased substantially after incorporating the candidate gene SNPs in the linkage analysis (by 0.46 Z and an order of magnitude in *P*-value). However, the absolute peak of the linkage signal is >16 cM from these candidate genes and the correlation between allele-sharing at the candidate SNPs and the allele-sharing due to linkage is not substantial. Therefore, it is clear that these candidate gene SNPs do not account for the complete linkage signal, although two of the SNPs could be contributing to the linkage signal. These SNPs (HTR1D(1080C > T) and OPRD1(80T > G)) could be in linkage disequilibrium with SNPs that explain a larger portion of the linkage signal. Note that the addition of the candidate gene SNPs did not reduce the width of the linkage peak and has introduced a

secondary linkage peak at OPRD1, that is, it appears that the region around OPRD1 may be contributing to a linkage signal that is distal of the D1S3721 signal. If these SNPs are indeed the only SNPs affecting liability in the region, then a portion of the linkage signal (Figure 1) is due to stochastically elevated ibd-sharing among affected relatives.

Association at HTR1D

To our knowledge, this is the first report of an association of HTR1D sequence variation in AN or in any other psychiatric illness. When this investigation was initiated, only two HTR1D SNPs were reported in the literature. For this reason, an effort was made to resequence the HTR1D gene locus. This resulted in the discovery of three novel SNPs, which were subsequently evaluated for statistical association with AN, along with one of the previously reported SNPs. These HTR1D SNPs were subsequently identified by other groups as putative *in silico* SNPs and have been given HGVBase SNP IDs (see Table 1). HTR1D(1080C>T) exhibited allele frequencies in the EAF control sample identical to the previously reported frequency,[37] and is associated with AN in the case : control design in our study with an OR of 2.63, the greatest OR identified in these analyses (Table 2). The level of significance is sufficient to withstand the effects of an intragenic Bonferroni correction. Two locus haplotypes containing HTR1D(1080C>T) were also found to be significantly associated with DSM-IV AN, although the four-locus HTR1D SNP haplotype was not associated (Table 3). However, HTR1D(1080C>T) was not found to be in significant transmission disequilibrium in a family-based design (Table 4), which may be attributable to the low power of the TDT test with alleles of low frequency.[50,51] The three other HTR1D SNPs genotyped in this analysis were found to exhibit significant transmission disequilibrium (Table 4) and are also in very significant LD with each other (Table 4 of the web supplement). Significant association results based on the case : control approach and using the transmission disequilibrium approach provide greater evidence for the involvement of the HTR1D gene in AN than would have been the case for either approach alone. HTR1D(1080C>T) is a silent mutation, while the three remaining SNPs are either 5′ or 3′ of the coding region. Therefore, the significant associations reported here are unlikely to affect the function of the 5HT1D receptor, but they may be associated with differences in HTR1D gene expression or in linkage disequilibrium with other polymorphisms at HTR1D or another linked locus that affects the expression of HTR1D.

Association at OPRD1

Two OPRD1 sequence variants have been reported in the psychiatric genetic literature.[38,39] Published molecular genetic studies of a silent OPRD1 polymorphism (921T>C) provide contradictory evidence that the delta opioid receptor is associated with heroin and alcohol dependence in case : control and trio association designs and to alcohol dependence in case : control and trio designs.[38,52] Significant allele frequency heterogeneity among six continental population groups was observed at this polymorphism and another reported OPRD1 sequence variant, a non-conservative amino-acid substitution (80T>G, Phe27Cys).[39] However, OPRD1 80G allele frequencies determined in the EAF sample (minor allele = 0.11) were nearly identical to the previously reported frequency (minor allele = 0.12) in European Americans.[39] The EAF control population consists of European-American individuals, the continental origin of which is not dissimilar to the composition of the AN sample. As this OPRD1(80T>G) has previously identified continental-level ethnic frequency heterogeneity,[39] this suggests that the AN proband and EAF samples studied here may be ethnically matched at a continental population level. This study found statistically significant frequency associations to the DSM-IV AN phenotype and a trend towards transmission disequilibrium at multiple OPRD1 SNPs using the case : control and transmission disequilibrium (TDT) designs, respectively. Statistically significant allelewise association to AN probands at three of the five OPRD1 SNPs was observed (Table 2). Two-locus and multilocus SNP haplotype frequencies containing OPRD1(47821A>G) were found to be significantly different between the AN proband and the EAF control sample (Table 3). In the TDT analysis, a trend ($P = 0.06$) for transmission disequilibrium using AN probands and parents was found at two OPRD1 SNPs (Table 4). It is possible that the results reported here reflect a role of the OPRD1 gene in AN if there is an effect of the associated alleles on gene expression of OPRD1.

Power of the sample

The odds ratios (ORs) associated with the most significant findings in this study were 2.63 (95% CI 1.21–5.75) at HTR1D(1080C>T) (allele 1 to allele 2) and 1.64 (95% CI 1.11–2.44) at OPRD1(47821A>G) (allele 2 to allele 1). This data set had insufficient power (<80%) to detect the significant associations using case–control and TDT association approaches, assuming effect sizes of 1.6–2.6 and the frequency of minor alleles observed. However, this study possessed a case sample size as large or larger than many AN case samples in the literature[53] and the control sample was screened to exclude obesity and lifetime Axis I psychiatric disorders. Using the structure of the family sample with significant TDT results at the three HTR1D SNPs, it would be necessary to have between 274 and 479 families to observe a significant association at a Type I error level of 0.05 and a Type II error level of 0.80, respectively. While the use of family-based data to test association and linkage of AN phenotype to candidate gene sequence variation provided additional statistical support for the

case–control association findings in this study, much larger samples will be required to confirm the observed associations using either case : control or family association designs to avoid Type II error. In addition, because the case samples in the case : control and transmission disequilibrium analyses used in this study were severely affected probands from multiplex families who meet rigorous AN criteria for inclusion in the study,[27] it will be important to use a significantly larger sample than the sample used in this study in any attempt to confirm the association findings at HTR1D and OPRD1 if the severity of the AN diagnosis is related to HTR1D or OPRD1 SNP allele frequency and/or if future AN samples are not as severely affected as the current case sample.

Multiple testing issues

These candidate gene analyses are part of ongoing analyses of a whole genome linkage scan and candidate gene investigation in eating disorders.[27,28,54] Review of the Human Genome Project assembly suggests that there is approximately one gene or expressed sequence every 120 000 bp in the chr1p region being evaluated. Another candidate gene locus in this region, the hypocretin receptor 1 (HCRTR1), has been evaluated at four intragenic SNPs using the case : control design as above and no association or trend of association to AN was identified. While multiple testing is known to inflate the number of false positives, the statistics are highly correlated due to the physical proximity of the markers analyzed. Therefore, a Bonferroni-type correction would be very conservative, and we do not report one here. However, because we are presenting the results of contingency analysis at two genes which do not exhibit detectable LD between them (Table 4 of the web supplement), the readers may bear in mind that an α value of 0.025 (or 0.017 if one includes HCRTR1) would be an appropriate threshold for significance. Applying this critical value to the contingency association results would have the effect of reducing the significant association results to those observed at HTR1D(1080C > T) and OPRD1(47821A > G). LD studies have been conducted with these samples on 72 additional SNPs in 31 additional candidate genes, which also raises the issue of multiple hypothesis testing. The exact nature of a statistical correction for this testing is difficult to determine,[55] as the statistical significance of significant candidate gene associations identified in these samples would need to be evaluated taking into consideration both statistical genetic information derived from the linkage analysis and neurobiological information regarding candidate genes.

Association with AN subtypes

Some of the case : control association results suggest that the association with these candidate gene SNPs may be stronger for one or another AN subtypes, restricting AN or restricting-purging AN. For example, when the heterogeneity analyses of HTR1D two-locus haplotypes containing HTR1D(1080C > T) were repeated for the AN subsamples separately, the significant findings for the full AN sample with the EAF control group were found to depend more on the restricting type AN subgroup than the restricting-purging type AN subgroup. For OPRD1, there is evidence to suggest that case : control findings differ with respect to AN subtype and location of the SNP within the OPRD1 locus, despite the reduction in power due to the reduced sample size. Specifically, case : control association findings are stronger for restricting AN at OPRD1 SNPs in exon 1 and IVS1 and for restricting-purging AN at OPRD1 SNPs in exon 3. These AN subtype findings, which are as significant as the overall AN findings, should be further explored in future studies. These findings are of particular interest because it is expected that the AN subtypes would exhibit less significant association because of the 50% reduction in case sample size (the AN proband sample is 55% restricting anorexic and 45% purging anorexic[27]).

Conclusion

The sequence variation at HTR1D and OPRD1 is of unknown function: the sequence variation evaluated at HTR1D either flanks the coding region (three of four SNPs) or is synonymous; the sequence variation at OPRD1 is intronic (three of five SNPs), 3′ flanking or nonsynonymous. It is not possible to state that the HTR1D and OPRD1 sequence variation that has been investigated and shown to exhibit significant association with AN and nominal association with the linkage results to AN is functionally responsible for the observed associations in the absence of functional clinical or *in vitro* correlates. The investigated sequence variation or sequence variation in LD with the investigated sequence variation may be functionally responsible for the observed associations. Further genetic evidence for the involvement of these receptors in AN derived from additional LD analyses may provide the necessary stimulus for functional studies of the effects of sequence variation on HTR1D and OPRD1 gene expression and for clinical studies of serotonergic and opioidergic function in AN. These additional LD analyses should utilize novel samples or additional linked sequence variation at HTR1D and OPRD1, or both, to define the region of maximal LD and identify candidate polymorphisms for functional analysis.

Current treatments for AN are symptomatic and often ineffective and no pharmacological agent has been established to be of benefit in the treatment of underweight people with AN.[56] Thus genetic studies may help identify new treatment targets. Future studies should investigate the role of the serotonin 1D and delta opioid receptors in the etiology of AN, potential differential association to subtypes of AN, and interactions between these receptors and other genes involved in serotonergic and opioidergic function and metabolism.

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