Neuropeptide Y Receptor Genes Are Associated With Alcohol Dependence, Alcohol Withdrawal Phenotypes, and Cocaine Dependence

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Background: Several lines of evidence in both human and animal studies suggest that variation in neuropeptide Y (*NPY*) or its receptor genes (*NPY1R, NPY2R* and *NPY5R*) is associated with alcohol dependence as well as alcohol withdrawal symptoms. Additional studies suggest that cocaine may affect *NPY* expression.

Methods: A total of 39 single nucleotide polymorphisms (SNPs) were genotyped across *NPY* and its 3 receptor genes in a sample of 1,923 subjects from 219 multiplex alcoholic families of European American descent recruited as part of the Collaborative Studies on the Genetics of Alcoholism (COGA) study. Family-based association analysis was performed to test the primary hypothesis that variation in these genes is associated with alcohol dependence. Secondary analyses evaluated whether there was an association of these SNPs with symptoms of alcohol withdrawal, cocaine dependence, or comorbid alcohol and cocaine dependence.

Results: Although variations in *NPY* itself were not associated with these phenotypes, variations in 2 NPY-receptor genes were. SNPs in *NPY2R* provided significant evidence of association with alcohol dependence, alcohol withdrawal symptoms, comorbid alcohol and cocaine dependence, and cocaine dependence (all $p < 0.03$). Haplotype analyses strengthened the evidence for these phenotypes (global $0.0004 < p < 0.005$). SNPs in *NPY5R* demonstrated significant association with alcohol withdrawal characterized by seizures ($p < 0.05$).

Conclusion: These results indicate that sequence variations in *NPY* receptor genes are associated with alcohol dependence, particularly a severe subtype of alcohol dependence characterized by withdrawal symptoms, comorbid alcohol and cocaine dependence, and cocaine dependence.

Key Words: Alcoholism, Withdrawal, Cocaine Dependence, Neuropeptide Y, Genetic Association.

ALCOHOL DEPENDENCE IS a common disorder affecting 4 to 5% of the United States population at any given time, (Li et al., 2007) with a lifetime prevalence of 12.5% (Hasin et al., 2007). Family, twin, and adoption studies have consistently demonstrated a substantial genetic contribution to disease etiology (Cloninger et al., 1981; Heath et al., 1997; Kendler et al., 1994; McGue, 1999; Pickens et al., 1991). Recent human studies have identified several genes associated with alcohol dependence, including *GABRA2* (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005), *ADH4* (Edenberg et al., 2006; Guindalini et al., 2005; Luo et al., 2005b), *GABRG3* (Dick et al., 2004), *CHRM2* (Luo et al., 2005a; Wang et al., 2004), *NFKB1* (Edenberg et al., 2007), *OPRK1* and *PDYN* (Edenberg et al., 2008; Xuei et al. 2006), and *TAS2R16* (Hinrichs et al., 2006).

A complementary approach to the identification of genes contributing to the risk for human alcoholism is the analysis of alcohol-related phenotypes in animal models. For example, the alcohol-preferring (P) and alcohol-nonpreferring (NP) rats have been shown to be an animal model of alcohol dependence (Files et al., 1992; Li et al., 1991). The P rats voluntarily consume large amounts of alcohol for its pharmacological effects, work hard to obtain alcohol, and demonstrate tolerance when allowed to drink freely (Carr et al., 1998; Li et al., 1993; Murphy et al., 2002). Using data from this animal model, strong evidence of linkage for alcohol preference was found on rat chromosome 4, in a region which included several positional candidate genes including the gene for α-synudein (*SNCA*) (Carr et al., 1998; Liang and Carr, 2006; Liang et al., 2003).

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Subsequent human studies have demonstrated that variation in *SNCA* does not contribute to the overall risk of alcohol dependence, but is associated with the phenotype of alcohol craving that may be related to the preference in rats (Bonsch et al., 2004, 2005a,b,c; Foroud et al., 2007).

The same region on rat chromosome 4 also includes the candidate gene neuropeptide Y (*Npy*; Spence et al., 2005). Numerous studies in animal models have shown that NPY plays a role in alcohol preference and consummatory behavior (Badia-Elder et al., 2001, 2003, 2007; Caberlotto et al., 2001; Cowen et al., 2004; Ehlers et al., 1998; Kimpel et al., 2007; Pandey et al., 2003; Schroeder et al., 2003; Spence et al., 2005; Tecott and Heberlein, 1998; Thiele and Badia-Elder, 2003; Thiele et al., 2004a; Thorsell, 2007). For example, infusion of NPY reduces ethanol intake in P rats (Gilpin et al., 2003), and *Npy*-deficient mice consume more alcohol than wild-type mice (Thiele et al., 1998). It has also been shown that cocaine administered in Sprague-Dawley rats reduced *Npy* mRNA in the prefrontal cortex, and reduced NPY-like (NPY-LI) immunoreactivity in the cingulate cortex and nucleus accumbens (Wahlestedt et al., 1991). In the same rat strain, NPY-LI immunoreactivity was found to be expressed in the dentate gyrus, a region of the hippocampus where this expression is not typically found, after a cocaine-induced seizure (Goodman and Sloviter, 1993). These results suggest that NPY may also play a role in response to cocaine.

NPY is a highly conserved 36-amino-acid peptide (de Quidt and Emson, 1986; Sundler et al., 1986) and has multiple functions including anxiolytic regulation (Heilig and Thorsell, 2002), food intake stimulation (Clark et al., 1984; Jolicoeur et al., 1991; Zarjevski et al., 1993), and neuronal excitability (Woldbye et al., 1996). NPY is abundant in the cortex, striatum, nucleus accumbens, amygdala, and hypothalamus (Badia-Elder et al., 2007; Gray and Morley, 1986; Heilig and Widerlov, 1995; Spence et al., 2005).

A functional single nucleotide polymorphism (SNP) in *NPY*, Leu7Pro (rs16139) (Karvonen et al., 1998), has been extensively studied for its association with alcohol dependence, consumption, and withdrawal symptoms; however, results have been inconsistent. Independent studies have found that the Pro7 allele is more frequent in alcohol dependent individuals than in controls (Kauhanen et al., 2000; Lappalainen et al., 2002), more common in individuals with late onset of alcohol dependence than in those with early onset (Mottagui-Tabar et al., 2005), and more common in alcoholics experiencing severe withdrawal symptoms and higher daily alcohol consumption (Koehnke et al., 2002). In Finnish men, the same allele appeared weakly associated with higher weekly consumption of alcohol (Kauhanen et al., 2000). In contrast, the Pro7 allele when found in heterozygous form was less common among alcoholics than social drinkers (Ilveskoski et al., 2001). Other studies found no significant differences in allele frequency between alcohol dependent individuals and controls of Finnish, Swedish or German origin (Hu et al., 2005; Mottagui-Tabar et al., 2005; Zhu et al., 2003; Zill et al., 2008). Studies of human alcoholics drinking more than 80 g of ethanol per day for most of their adult lives demonstrated a decrease in NPY immunoreactivity in the amygdala (Pluzarev and Crews, 2007) and decreased gene expression of *NPY* in the frontal and motor cortices (Mayfield et al., 2002).

NPY has also been associated with withdrawal from alcohol. Koehnke et al. (2002) reported that the Pro7 allele is more common in alcohol dependent individuals with delirium tremors (DT) or who have experienced withdrawal with seizures than in alcoholic dependent individuals with mild withdrawal symptoms. Okubo and Harada (2001) reported association of the 5671C/T polymorphism (rs5574) in *NPY* with alcohol dependent individuals who experienced withdrawal with seizures. Several animal models have also demonstrated that withdrawal from ethanol reduces NPY expression (Bison and Crews, 2003; Thiele and Badia-Elder, 2003; Thiele et al., 2004b; Thorsell, 2007). For example it has been shown that ethanol withdrawal produced significant reduction in NPY protein levels in the central and medial nuclei of the amygdala, cortical, and hypothalamic structures in rats (Roy and Pandey, 2002). Further evidence shows that intracerebroventricular administration of NPY in Wistar rats in withdrawal significantly decreased the withdrawal scores of the rats (Woldbye et al., 2002).

Three G protein-coupled *NPY* receptor genes, *NPY1R*, *NPY2R*, and *NPY5R* have been shown to be associated in animals with alcohol preference (Eva et al., 2006; Thiele and Badia-Elder, 2003; Thorsell and Heilig, 2002) and withdrawal (Bison and Crews, 2003; Thiele et al., 2004b; Thorsell et al., 2007; Valdez and Koob, 2004; Woldbye et al., 2002). These 3 genes are located on chromosome 4q31-q32 (Lutz et al., 1997; Wraith et al., 2000), near the edge of a broad linkage peak for the risk for alcohol dependence identified in the Collaborative Study on the Genetics of Alcoholism (COGA) sample (Reich, 1996; Reich et al., 1998; Williams et al., 1999).

Given that *NPY* modulates consummatory behavior and the positive, rewarding properties associated with alcohol consumption (Thiele et al., 2004b), and the somewhat inconsistent results from human studies, the NPY system seems a good candidate to study in a population of densely affected, alcohol dependent families. We have performed a detailed evaluation of the NPY system, including *NPY* and its receptor genes *NPY2R*, *NPY1R* and *NPY5R*, in relation to alcohol dependence with and without withdrawal symptoms, and cocaine dependence. The latter was included because of evidence that *NPY* may be involved in cocaine-seeking behavior and in the response to cocaine.

MATERIALS AND METHODS

Association Sample

COGA is an ongoing multi-site study that has recruited families at centers across the United States. To limit heterogeneity a sample of 1,923 European American subjects from 219 families was used in the present analysis; they were recruited at Indiana University, State University of New York Downstate Medical Center, University of Connecticut, University of Iowa, University of California/San

Diego, and Washington University, St. Louis. This study was approved by the institutional review boards of all participating institutions. Each family was ascertained through a proband seeking treatment at an alcohol treatment program (Begleiter et al., 1995; Foroud et al., 2000; Nurnberger et al., 2004; Reich et al., 1998).

A poly-diagnostic instrument, the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994; Hesselbrock et al., 1999) was administered to probands and their families. The families that participated in the genetic phase of this study had at least 3 first-degree relatives who met both lifetime DSM-IIIR criteria for alcohol dependence (American Psychiatric Association, 1987) and lifetime Feighner criteria (Feighner et al., 1972) for definite alcoholism. Further details of the ascertainment and assessment can be found elsewhere (Begleiter et al., 1995; Foroud et al., 2000; Reich et al., 1998).

Phenotypes

We initially tested for an association between the 4 genes and alcohol dependence as defined by DSM-IV criteria (American Psychiatric Association, 1994). Secondary hypotheses based upon both the human and animal literature included alcohol withdrawal symptoms, such as seizures and cocaine dependence. The number of affected subjects analyzed for each phenotype is shown in Table 1. Using items from the SSAGA, 2 measures of alcohol withdrawal were analyzed. The first was whether an individual ever experienced any of 9 problems (shakes, sleeplessness, anxiety, sweating, fast heart beat, nausea/vomiting, physically weak, headaches, or seeing/hearing things that were not there) after having stopped, cut down, or gone without drinking. Subjects were classified as affected if they met 3 criteria: (1) responded affirmatively to having at least one of the problems, (2) took any medication/drug to avoid any of these problems (or to make them go away), and (3) were classified as DSM-IV alcohol dependent. The medication requirement was included so as to more closely approximate the 'severe' withdrawal of Koehnke et al. (2002). Subjects were coded as unaffected if they were classified as DSM-IV dependent but did not experience any of the 9 symptoms. All other subjects were considered unknown (Table 1). This phenotype is referred to as severe withdrawal.

The second alcohol withdrawal phenotype classified as affected those subjects who met criteria for DSM-IV alcohol dependence and also responded affirmatively to at least 1 of 2 questions: (1) "When you stopped, cut down, or went without drinking, did you have fits, seizures, or convulsions, where you lost consciousness, fell to the floor, and had difficulty remembering what happened" or (2) "Did you have the DT's [delerium tremens], where you were very confused, extremely shaky, felt very frightened or nervous, or saw things that weren't really there when you stopped, cut down, or went without drinking?" Subjects who were classified as DSM-IV and responded negatively to both questions were considered unaffected. All other subjects were considered unknown (Table 1). This phenotype is termed withdrawal with seizures.

Because of the reported relationship between NPY-LI expression and cocaine-seeking behavior (Boutrel et al., 2005; Menyhert et al., 2007; Wahlestedt et al., 1991), we tested for an association with cocaine dependence, defined by DSM-IIIR criteria. Due to the large number of cocaine dependent individuals who are comorbid for alcohol dependence (Table 1), we also analyzed individuals who met criteria for both DSM-IV alcohol dependence and cocaine dependence. Individuals who were neither alcohol dependent nor cocaine dependent were classified as unaffected for this phenotype. All other individuals were considered unknown. As most cocaine dependent individuals were also alcohol dependent (208 out of 255), there was not a sufficient sample to analyze cocaine dependence excluding alcohol dependence ($n = 47$). To avoid confounding cocaine dependence and alcohol dependence, whenever evidence of association was found with both alcohol dependence and cocaine dependence ($p < 0.05$), an additional analysis was performed which included as affected only those individuals who met criteria for DSM-IV alcohol dependence but did *not* meet DSM-IIIR criteria for cocaine dependence (Table 1). All cocaine dependent individuals who were not alcohol dependent ($n = 47$) were classified as unknown for this alcohol-only phenotype. Thus, unaffected individuals were defined as those who were neither alcohol nor cocaine dependent.

SNP Genotyping

NPY is located on 7p15.1 and is 7.7 kb in size; *NPY2R* is on 4q32.1 and is 8.6 kb in size. The other 2 receptor genes, *NPY1R* and *NPY5R*, are only 8kb apart, span 28 kb on 4q32.2 and were analyzed together. SNPs distributed throughout the 4 genes were selected from public databases, primarily dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), based on their spacing and available allele frequencies. At the time some SNPs were selected, allele frequencies were not available. To determine allele frequencies and to test the quality of the assays, SNPs were genotyped in 2 sets of samples, each consisting of 40 unrelated individuals from the Coriell European- and African-American diversity samples; only SNPs in Hardy–Weinberg equilibrium in both test groups were genotyped on the COGA sample.

A total of 39 SNPs were genotyped in the sample, including the known coding SNP Leu7Pro in *NPY* (rs16139) located in exon 2. Genotyping was done using a modified single nucleotide extension reaction, with allele detection by mass spectrometry (Sequenom MassArray Sysem; Sequenom, San Diego, CA). All SNP genotypes

Table 1. Phenotypic Characteristics of Genotyped Individuals

Phenotypes		No. affected (%)	No. unaffected (%)	No. unknown (%)
Alcohol dependence		753 (39%)	1047 (55%)	123[a] (6%)
Withdrawal—severe	Alcohol dependence + withdrawal symptoms and medication	176 (9%)	292 (15%)	1455 (76%)
Withdrawal—seizures	Alcohol dependence + withdrawal with seizures	105 (5%)	648 (34%)	1170 (61%)
Cocaine dependence	With or without alcohol dependence	255 (13%)	1545 (80%)	123 (6%)
Alcohol dependence without cocaine dependence	Alcohol dependence no cocaine dependence	545 (28%)	1000 (52%)	378 (20%)
Alcohol dependence with cocaine dependence	Alcohol dependence plus cocaine dependence	208 (11%)	1000 (52%)	715 (37%)
Cocaine dependence without alcohol dependence[b]	Cocaine dependence no alcohol dependence	47 (2%)	1000 (52%)	876 (46%)

[a]There are 123 individuals who did not complete a SSAGA interview and therefore are classified as unknown for all phenotypes.
[b]Not analyzed for lack of power.

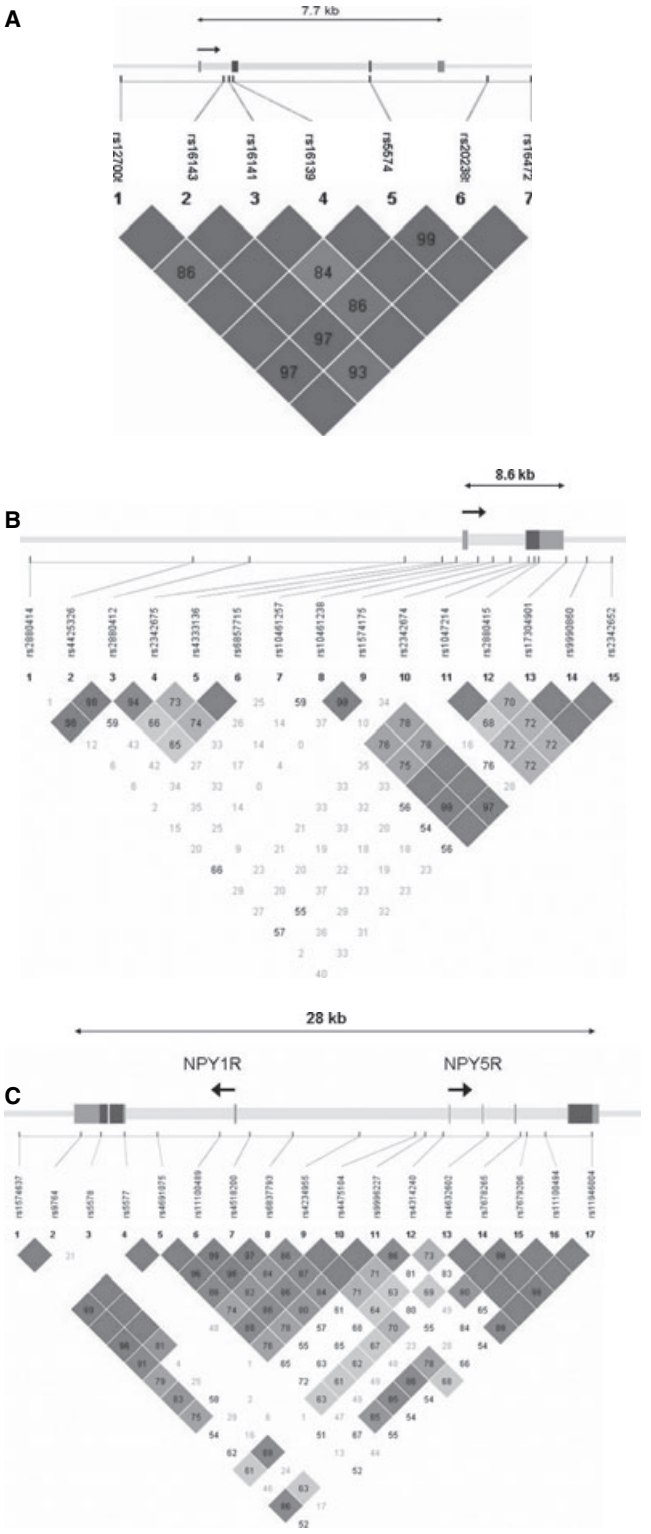

Fig. 1. (**A**) Genomic structure of *NPY*. The direction of transcription and the exons are indicated by the arrow and rectangular block, respectively. Pairwise linkage disequilibrium (LD) estimates, genotyped in the COGA sample, are given as D′. Darkly shaded boxes illustrate strong evidence of LD, defined as a pair of SNPs with the 1-sided upper 95% confidence bound on D′ of 0.98 and the lower bound above 0.70. Lightly shaded boxes indicate lower LD. (**B**) Genomic structure of *NPY2R*. The direction of transcription and the exons are indicated by the arrow and rectangular block, respectively. Pairwise LD estimates, genotyped in the COGA sample, are given as D′. Symbols are as in (**A**). (**C**) Genomic structure of *NPY1R/NPY5R*. The direction of transcription and the exons are indicated by the arrow and rectangular block, respectively. Pairwise LD estimates, genotyped in the COGA sample, are given as D′. Symbols are as in (**A**).

were checked for Mendelian inheritance using PEDCHECK (O'Connell and Weeks, 1998). Marker allele frequencies and heterozygosities were computed using USERM13 (Boehnke, 1991). Markers were tested for Hardy–Weinberg equilibrium using Haploview (Barrett et al., 2005). No marker deviated significantly ($p < 0.01$) from Hardy–Weinberg equilibrium. Linkage disequilibrium (LD) measured by D′ is depicted for *NPY*, *NPY2R*, and the *NPY1R/NPY5R* cluster in Fig. 1*A* and 1*C*.

Statistical Analysis

To ensure that the genotyped SNPs adequately covered the genes under consideration, LD was computed using the program Haploview (Barrett et al., 2005). An independent evaluation was performed using the program Tagger (de Bakker et al., 2005) to calculate the fraction of all SNPs in each region analyzed by HapMap [with minor allele frequency (MAF) >0.10] that were in LD ($r^2 \geq 0.80$) with the SNPs we genotyped.

The pedigree disequilibrium test (PDT) (Martin et al., 2001), as implemented in the program UNPHASED (Dudbridge, 2003), was used to test whether the qualitative phenotypes in the extended, multiplex COGA pedigrees were associated with the genotyped SNPs. The PDT-average statistic, which weighs each family equally in computing the overall test statistic, was the statistic of interest for each phenotype.

To reduce the scope of hypothesis testing, multi-SNP haplotypes were constructed only when 2 or more phenotypes were significant ($p \leq 0.05$) within any 1 gene. To avoid constructing haplotypes based on SNPs providing redundant information, only SNPs with low pairwise LD ($r^2 < 0.50$) were used in haplotype analysis. Each haplotype was then examined to determine whether significant association results were due to the overtransmission of a particular haplotype to affected individuals or to the differential transmission of particular haplotypes to siblings discordant for the phenotype. Except for the severe withdrawal phenotype, haplotypes were estimated using phase-certain genotyped individuals in the program UNPHASED (Dudbridge, 2003). Due to the small number of both affected and unaffected subjects for the severe withdrawal phenotype, missing haplotypes were estimated using the EM algorithm. All haplotypes with a frequency less than 0.05 were omitted from association analyses.

RESULTS

Coverage of Variation in the Genes

To determine how well the genotyped SNPs represented the known variation (from the HapMap CEU database) in the regions of interest, we applied the program Tagger (de Bakker et al., 2005). Seven SNPs were genotyped across the 13 kb region containing *NPY*, extending 4 kb beyond each end of the gene (Table 2, Fig. 1A). The average r^2 of these 7 SNPs with all of the 27 known HapMap SNPs (MAF ≥ 0.10) in the 13 kb region was 0.96; $r^2 > 0.8$ for 96% of the SNPs. The Leu/Pro7 polymorphism in *NPY* (rs16139) had a MAF of 0.05.

Fifteen SNPs were genotyped across the 48 kb region containing *NPY2R*, extending 34 kb on the 5′ end and

Table 2. Association of *NPY* and NPY Receptor Genes and Study Phenotypes

Gene	Id	SNP	Position[a]	Location[b]	MAF[c]	Alcohol dependence[d]	Withdrawal—severe[d]	Withdrawal—seizures[d]	Cocaine dependence[d]
NPY	1	rs12700524	24,287,939	Upstream	0.13	0.79	0.77	0.52	0.81
	2	rs16143	24,291,113	Intron 1	0.25	0.70	0.80	0.80	0.20
	3	rs16141	24,291,284	Intron 1	0.49	0.26	0.41	0.46	0.19
	4	rs16139	24,291,404	Exon 2, Pro7Leu	0.05	0.84	0.68	0.41	0.91
	5	rs5574	24,295,658	Exon 3, Ser68	0.49	0.39	0.73	0.73	0.42
	6	rs2023890	24,299,272	Downstream	0.23	0.73	0.80	0.41	0.63
	7	rs161416472	24,300,594	Downstream	0.09	0.31	0.82	0.14	0.25
NPY2R	1	rs2880414	156,313,101	Upstream	0.29	0.27	0.89	0.37	0.38
	2	**rs4425326**[e]	156,326,686	Upstream	0.36	**0.03**	**0.03**	0.35	**0.02**
	3	rs2880412	156,331,429	Upstream	0.26	0.60	0.88	0.52	0.15
	4	rs2342675	156,344,373	Upstream	0.35	0.53	0.58	0.46	**0.03**
	5	rs4333136	156,347,499	Upstream	0.38	**0.02**	0.08	0.60	**0.004**
	6	**rs6857715**[e]	156,348,632	Promoter	0.37	**0.03**	**0.03**	0.92	**0.0005**
	7	rs10461257	156,350,454	Intron 1	0.28	0.35	0.31	0.37	0.93
	8	rs10461238	156,351,666	Intron 1	0.40	0.71	0.41	0.32	0.08
	9	rs1574175	156,353,162	Intron 1	0.12	0.83	0.85	0.94	0.28
	10	rs2342674	156,354,700	Exon 2, Leu53	0.01	0.38	1.00	0.32	0.06
	11	rs1047214	156,355,126	Exon 2, Ile195	0.46	0.34	0.69	0.78	0.40
	12	rs2880415	156,355,477	Exon 2, Ile312	0.47	0.28	0.49	0.81	0.18
	13	rs17304901	156,357,822	Downstream	0.27	0.35	0.18	0.31	**0.005**
	14	rs9990860	156,359,515	Downstream	0.22	0.72	0.82	0.28	0.21
	15	rs2342652	156,361,562	Downstream	0.48	0.81	0.12	0.61	0.76
NPY1R	1	rs1574637	164,461,542	Downstream	0.11	0.16	0.65	0.45	0.55
NPY1R	2	rs9764	164,464,855	3′ UTR	0.26	0.28	0.19	0.73	0.47
NPY1R	3	rs5578	164,465,939	Exon 3, Thr374Lys	0.01	0.89	0.32	0.32	0.78
NPY1R	4	rs5577	164,467,193	5′ UTR	0.02	0.87	0.94	0.65	0.79
NPY1R	5	rs4691075	164,468,935	Intron 1	0.12	0.15	0.63	0.38	0.67
NPY1R	6	rs11100489	164,472,262	Intron 1	0.10	0.25	0.85	0.54	0.75
NPY1R	7	rs4518200	164,473,872	Promoter	0.11	0.21	0.63	0.73	0.90
	8	rs6837793	164,476,185	Intergenic	0.11	0.24	0.51	0.56	0.73
	9	rs4234955	164,479,726	Intergenic	0.26	0.45	0.87	0.97	0.51
	10	rs4475104	164,482,736	Intergenic	0.11	0.67	0.33	**0.05**	0.56
NPY5R	11	rs9996227	164,483,251	Promoter	0.11	0.29	0.17	0.06	0.82
NPY5R	12	rs4314240	164,484,226	Promoter	0.11	0.87	0.20	**0.05**	0.34
NPY5R	13	rs4632602	164,486,579	Intron 2	0.12	0.42	0.33	**0.008**	0.75
NPY5R	14	rs7678265	164,488,364	Intron 3	0.09	0.22	0.55	**0.02**	0.33
NPY5R	15	rs7679206	164,488,684	Intron 3	0.23	0.09	0.21	0.36	0.86
NPY5R	16	rs11100494	164,489,703	Intron 3	0.07	0.89	0.38	0.75	0.81
NPY5R	17	rs11946004	164,492,153	Exon 4, Gly426	0.12	0.13	0.60	0.06	0.71

NPY, neuropeptide Y; SNP, single nucleotide polymorphism.
[a]Position in nucleotides, from NCBI Human Genome Assembly (version 36.2)
[b]Position within or near gene.
[c]Minor allele frequency calculated from the COGA dataset.
[d]Pedigree disequilibrium test average statistic *p*-value. Bold values are significant ($p \leq 0.05$) association SNPs.
[e]SNPs used in haplotype analysis.

4 kb on the 3′ end (Table 2). The average r^2 of the 8 HapMap SNPs among these with all of the 83 known HapMap SNPs (MAF ≥ 0.10) in the 48 kb *NPY2R* region was 0.85; $r^2 > 0.8$ for 81% of the SNPs. Because 7 of the 15 SNPs we genotyped were not in the HapMap database, this is the lower boundary of coverage. *NPY2R* has weak LD along the 3′ end and stronger LD between SNPs 8-15 on the 5′ end of the gene, as can be seen in Fig. 1B.

Seventeen SNPs were genotyped across the 31 kb region containing *NPY1R* and *NPY5R*, extending 3 kb on the 3′ end of *NPY1R*. The average r^2 of 11 SNPs of the 17 SNPs genotyped on *NPY1R/NPY5R* with all of the 21 known HapMap SNPs (MAF ≥ 0.10) in the 31 kb region was 0.80; $r^2 > 0.5$ for 81% of the SNPs and $r^2 > 0.8$ for 67% of the SNPs (Table 2, Fig. 1C). Again, this is the lower bound of coverage because we genotyped 6 additional SNPs that could not be evaluated.

Association Results

Results of association analyses are provided in Table 2. None of the SNPs in *NPY*, including the Leu7Pro coding SNP rs16139, or in *NPY1R* were associated with any of the phenotypes.

One SNP in the promoter region of *NPY2R* (rs6857715) and 2 SNPs further upstream of the gene (rs4333136 and rs4425326) were associated with alcohol dependence ($p < 0.03$), and 2 of these with the secondary phenotype of severe withdrawal (Table 2). Five SNPs in this gene, the same

3 plus rs2342675 and rs17304901, were associated with cocaine dependence (Table 2). The same 3 SNPs were also associated with comorbid alcohol and cocaine dependence (rs6857715, $p = 0.006$; rs4333136, $p = 0.02$; rs4425326, $p = 0.03$; all other SNPs were nonsignificant ($p > 0.07$), data not shown). To determine whether the significant evidence of association for alcohol dependence was due to the subset of affected individuals who also met criteria for cocaine dependence (208 of the 753; Table 1), the analysis was repeated using only alcohol dependent individuals who were not cocaine dependent; there was no significant association of alcohol-only dependence with any of the SNPs ($p > 0.08$, data not shown).

Due to the consistency of the association results with the same set of 3 SNPs, we constructed haplotypes using only SNPs rs4425326 and rs6857715 ($r^2 = 0.18$); the high r^2 (0.98) between SNPs rs4333136 and rs6857715 made use of both redundant. The global haplotype test and several individual haplotypes were significantly associated with alcohol dependence, alcohol dependence with severe withdrawal, alcohol dependence in the absence of cocaine dependence, comorbid alcohol and cocaine dependence, and cocaine dependence (all global haplotypes $p < 0.04$; Table 3). For all 5 phenotypes, the most frequent haplotype, C-C was overtransmitted to affected individuals. The complementary, second-most frequent haplotype, T-T, was overtransmitted to individuals who did not meet the criteria.

Four SNPs in *NPY5R* were associated with the secondary phenotype of withdrawal with seizures: rs4475104, rs4314240, rs4632602, and rs7678265 ($p \leq 0.05$). SNPs rs9996227 and rs11946004 approached significance ($p \leq 0.06$).

DISCUSSION

This study is the first extensive examination of the association between the NPY system and alcohol dependence, and alcohol withdrawal and cocaine dependence. We took a systems approach and analyzed not only the *NPY* gene but also 3 of its receptor genes. The strongest association we found was between a haplotype in *NPY2R* with alcohol dependence as well as with several subphenotypes, including alcohol withdrawal symptoms, alcohol dependence without comorbid cocaine dependence, comorbid alcohol and cocaine dependence, and cocaine dependence. For each of the phenotypes analyzed, the same haplotype was preferentially transmitted to alcohol dependent individuals, with the strongest association being with all alcohol dependent individuals (the numerically largest group) and those suffering from alcohol withdrawal. Due to ascertainment criteria for the sample, we had insufficient power to test for an association of *NPY2R* and cocaine dependence in subjects who were not also alcohol dependent. Our results suggest that sequence variations in the NPY system, specifically in *NPY2R*, are associated with alcohol dependence characterized by severe withdrawal, as was reported in humans by Koehnke et al. (2002) and Okubo and Harada (2001) and by numerous findings in the animal literature (for a review, see Thiele et al., 2004b).

Neither *NPY1R* nor *NP5YR* was associated with alcohol dependence or cocaine dependence. However, *NPY5R* was associated with the phenotype of alcohol withdrawal with seizures, representing a small but severely affected subset of alcoholics.

We found no association of alcohol dependence with any SNPs in *NPY*, which is consistent with some previously reported results (Hu et al., 2005; Mottagui-Tabar et al., 2005; Zhu et al., 2003). The bulk of evidence for a role of the entire NPY system in alcohol-related phenotypes comes from animal models. While alcohol preference and consumption in rats and mice mimic similar traits of alcohol dependence in humans, their equivalence is still unclear (Crabbe, 2007). Our examination of the secondary phenotype of alcohol withdrawal provides another means to examine the nature of the association of these SNPs in the NPY system with alcohol dependence and provide important insights regarding disease heterogeneity.

Although our primary hypothesis was that genes in the NPY system were associated with alcohol dependence, we analyzed additional phenotypes related to alcoholism that were suggested by the literature. The withdrawal phenotypes each consist of a subset of the alcohol dependent subjects, as does the phenotype of comorbid alcohol and cocaine dependence; thus, they are not independent phenotypes, but phenotypes nested within alcohol dependence, analyzed to better understand what aspect of alcoholism is most affected by these genes. The cocaine dependence phenotype was considered a secondary analysis, and while it includes many subjects who also meet criteria for alcohol dependence, it is not

Table 3. Association Analysis of Haplotypes in *NPY2R*, All Entries Are *p* Values Using the Pedigree Disequilibrium Test, Average Statistic

rs4425326 Nucleotide	rs6857715 Nucleotide	Alcohol dependence	Severe withdrawal	Alcohol dependence without cocaine dependence	Comorbid alcohol with cocaine dependence	Cocaine dependence
C	**C**	**0.0002**	**0.0006**	**0.01**	**0.002**	**0.002**
C	T	0.32	0.27	0.50	0.28	0.25
T	C	0.19	0.15	0.29	0.52	0.62
T	T	**0.006**	**0.009**	0.08	**0.01**	**0.02**
Global test		**0.0004**	**0.0009**	**0.04**	**0.005**	**0.005**

Bold indicates $p \leq 05$.

a nested subset. Therefore, we are testing 2 phenotypes (alcohol dependence and cocaine dependence) and we have considered the strength of our association results if we were to apply a conservative Bonferroni correction ($0.05/2 = 0.025$). Applying this correction, we would still identify 1 SNP in *NPY2R* which is significantly associated with alcohol dependence and with comorbid alcohol and cocaine dependence, 2 SNPs in *NPY5R* which are significantly associated with withdrawal with seizures and 4 SNPs in *NPY2R* that are significantly associated with cocaine dependence. The results from haplotype analyses of *NPY2R* are even stronger (Table 3).

There are several strengths for this study. The sample is based on 1,923 individuals from 219 extended families, with a wealth of reliable and valid information obtained on each individual through the well-characterized SSAGA (Bucholz et al., 1994; Hesselbrock et al., 1999). This large sample was limited to European-Caucasian, non-Hispanic families, thus limiting heterogeneity of the haplotypes used in analyses. The use of family-based association tests reduced potential confounding from population stratification. Finally 39 SNPs were genotyped across *NPY* and its 3 receptor genes on chromosome 4, *NPY2R*, *NPY1R*, and *NPY5R*, all with moderate LD to establish extensive coverage of all genes.

In summary, using a family-based association test in extended alcoholic pedigrees, we found evidence of association of SNPs in the NPY receptor genes *NPY2R*, and *NPY5R* with alcohol dependence, comorbid alcohol and cocaine dependence, alcohol withdrawal, and cocaine dependence phenotypes which were identified previously in the animal literature. These results indicate that sequence variations in *NPY* receptor genes are associated with alcohol dependence, particularly a severe subtype of alcohol dependence characterized by withdrawal symptoms, comorbid alcohol and cocaine dependence, and cocaine dependence.

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