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Variation in *NGFB* is Associated with Primary Affective Disorders in Women

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Abstract

Affective disorders (AFDs) are highly comorbid with substance dependence (SD) and both are genetically influenced. However, the specific etiology of the comorbidity is not well understood. We genotyped an array of 1,350 single nucleotide polymorphisms (SNPs) in or near 130 genes in 868 European-Americans (EAs), including 182 individuals with primary AFDs (PAFDs), 214 with SD comorbid with AFD (CAFD), and 472 screened controls. *NGFB*, which encodes nerve growth factor β and was represented in the array by 15 SNPs, showed the strongest evidence of association, but only among women with PAFDs. Six of the SNPs showed a nominally significant association with PAFDs in women (P s = 0.0007–0.01); three (rs2856813, rs4332358, and rs10776799) were empirically significant based on 1,000,000 permutations (P s = 0.008–0.015). Seven haplotypes were significantly associated with PAFDs in women (P s = 0.0014–0.01), of which six were significant based on empirical permutation analysis (minimal P = 0.0045). Four diplotypes were significantly associated with PAFDs in women (global P s = 0.001–0.01). The specific diplotype GG-TC, reconstructed from rs2856813 and rs6678788, showed the strongest evidence of association with PAFDs in women (OR = 4.07, P = 4.2E-05). No SNPs or haplotypes were associated with PAFDs in men or with CAFDs in either sex. We conclude that variation in *NGFB* is a risk factor for PAFDs in women, but not for CAFD.

Keywords

nerve growth factor β ; *NGFB*; affective disorder; substance dependence; association study; sex-specific

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INTRODUCTION

Affective disorders (AFDs), characterized by dramatic changes or extremes, or other dysregulation of mood, are highly comorbid with substance dependence (SD). In the U.S. general population, the 12-month prevalence of AFDs and SD were 9.3% and 4.1%, respectively. The observed comorbidity was substantial: the 12-month prevalence of any AFD among survey respondents with diagnosed SD was 29.2%, and that of any SD among survey respondents who were also diagnosed with any AFD was 12.9% [Grant et al., 2004].

Although there appears to be a strong relationship between AFDs and SD, the biological nature of this relationship is not very clear. SD may increase risk for AFDs, AFDs may increase risk for SD, or they may share a common etiology. Compared to primary AFDs (PAFDs, defined here to mean those without comorbid SD), AFDs comorbid with SD (CAFDs) have an earlier age of onset, greater severity of symptoms, worse prognosis, poorer response to treatment, greater functional impairment, and greater risk of suicidal ideation [Davis et al., 2008]. Although on this basis it has been hypothesized both that PAFDs and CAFDs result from entirely different processes [Schuckit, 2006], the issue remains controversial [Markou et al., 1998]. In fact, PAFDs and CAFDs may each have unique causative risk factors as well as factors that overlap with one another. Genetic epidemiologic studies have demonstrated that AFDs and SD are individually heritable; the estimated heritability of bipolar disorders (BPs) ranged from 60–80% [Lichtenstein et al., 2009], and the estimated heritability of major depression (MD) and SD were both ~40% [Fu et al., 2002; Uhl and Grow, 2004] (although substantially higher heritability has been observed for some SD traits). However, it is difficult to identify genes that influence risk for AFDs or the comorbidity of AFDs with SD, in part because both AFDs and SD are complex disorders (i.e., they are influenced by environmental factors and numerous genes that interact with one another).

Our study examined 130 genes for their association to PAFDs and CAFDs by case-control association analysis using a candidate-gene array. Based on the results of that data mining effort, we focused on the gene encoding nerve growth factor beta (*NGFB*).

NGFB is of interest in this regard for a number of reasons. First, *NGFB* showed the strongest evidence of association (albeit only in female patients with PAFDs) among the 130 candidate genes in the initial data mining analysis. Second, the functions of the *NGFB* protein are related to the neurotrophic hypotheses of AFD development [Bersani et al., 2000]. Nerve growth factor (NGF), a member of the neurotrophin family, consists of 3 subunits (alpha, beta, and gamma), apparently with the beta unit (*NGFB*) being solely responsible for the biological activity of NGF [Barde, 1990]. Binding of *NGFB* to its receptor TrkA leads to activation of three signaling pathways, PI3K/Akt, Ras/raf/ERK, and PLC/PKC [Sofroniew et al., 2001]. Activation of these pathways is essential for the development, patterning, and maintenance of the nervous system. These pathways are also targets of mood stabilizers [Chen and Manji, 2006; Wada, 2009]. In addition, *NGFB* is regulated by testosterone [Katoh-Semba et al., 1990; Wright et al., 1987], which is relevant in light of the fact that women have a significantly higher lifetime risk of AFDs than men. The odds ratio of AFDs in females compared with males ranges from 1.6–2.2 [Seedat et al., 2009]. Finally, *NGFB* is closely associated to stress-related AFDs in animal models [Alleva and Santucci, 2001; Aloe et al., 2002; Marais et al., 2008].

METHODS

Subjects

European-American (EA) subjects were recruited at the University of Connecticut Health Center, Yale University School of Medicine, Brown University School of Medicine, and the Medical University of South Carolina. The institutional review board at each of the participating institutions approved the study protocol and consent processes. After subjects received a complete description of the study, they provided written informed consent to participate. Eight hundred sixty-eight unrelated subjects were included, of whom 396 had a lifetime AFD diagnosis (included 182 PAFDs and 214 CAFDs) and 472 were healthy controls. In the PAFD sample set, 167 of 182 subjects were diagnosed with MD, 3 with dysthymia, and 12 with seasonal depression; 11 have MD with anxiety disorders, five have MD with posttraumatic stress disorder, two have MD with obsessive compulsive disorder, and two have MD with social phobia. In the CAFD sample set, the subjects with affective disorders included 46 subjects with BP and 168 with MD. Various substances were abused by individuals in the CAFD group. The characteristics of the sample are shown in Table I.

Cases met lifetime DSM-III-R or DSM-IV diagnostic criteria for AFDs with or without comorbid SD (including dependence on drugs or alcohol), based on a structured interview, as described below. DSM-IV defines independent mood disorder as occurring in the absence of current substance use, medical illness, or bereavement. PAFD subjects were assessed for lifetime SD and met diagnostic criteria for independent AFDs without SD. All CAFDs were differentiated from PAFDs by virtue of the former having occurred in the context of substance dependence (rather than being accounted for by a medical illness or bereavement). Healthy controls were recruited by advertisement, and screened to exclude individuals with major Axis I psychiatric disorders, including SD, mood disorders, major anxiety disorders, and psychotic disorders. Diagnoses were made using the Structured Clinical Interview for DSM-III-R (SCID-III-R) [Spitzer et al., 1992] or DSM-IV (SCID-IV) [First et al., 1997], the Computerized Diagnostic Interview Schedule for DSM-III-R (CDIS-R) [Ross et al., 1995], or the Semi-structured Assessment for Drug Dependence and Alcoholism [SSADDA] [Pierucci-Lagha et al., 2005].

The self-identified ethnicity of case and control subjects was confirmed using a set of ancestry-informative markers (AIMs).

Markers and Genotyping

DNA was extracted from immortalized cell lines, blood, or saliva. A total of 1,350 SNPs within or near 130 candidate genes in 868 samples were genotyped using the Illumina GoldenGate Assay manufacturer's methods (Illumina Inc., San Diego, CA, USA), as described previously [Hodgkinson et al., 2008]. In addition, 113 AIMs were included in the array.

Markers were excluded if they deviated significantly from Hardy-Weinberg equilibrium (HWE) expectations ($P < 0.001$) in controls or if they had a minor allele frequency (MAF) < 0.025 , or a SNP call rate $< 95\%$.

Statistical Analysis

Data Mining: 130-Candidate-Gene Association Analysis—The chi-square trend model in JMP Genomics 3.1 software (SAS Institute Inc. Cary NC) was used to analyze the association of the 130 candidate genes (1,350 SNPs) to AFDs, PAFDs, and CAFDs. We performed 100,000 permutations to estimate empirical P -values and to address the issue of

multiple testing based on the full set of 1,350 SNPs. Statistical significance was defined by an empirical $P < 0.05$.

NGFB Association Analysis

Single-Marker Association Analysis: Fifteen SNPs, spanning 53,747 bp on chromosome 1 and covering the full length of *NGFB* (Table II), were analyzed using Haploview 4.1 software in the same case and control samples. The LD relationships of these 15 SNPs are displayed in Figure 1. Genotype-phenotype association analyses were performed by comparing the allele frequency distributions of individual SNPs between cases and controls with χ^2 tests. χ^2 tests were used to test for deviation from HWE with a cut-off of $P = 0.05$ (for the single-marker analysis).

Haplotype Association Analysis: Linkage disequilibrium (LD) values between SNP pairs and haplotype association were determined with Haploview version 4.1. Permutation tests with 1,000,000 permutations were used to evaluate significance in this analysis based on 15 single markers and haplotypes.

Diplotype Association Analysis: Diplotypes, also known as multi-locus genotype patterns, were constructed from SNP 9 (rs2856813), SNP10 (rs6678788) SNP 11 (rs4529705), and SNP12 (rs6537860). These SNPs were selected for inclusion in diplotype analyses based on their haplotype block structure (Figure 1) and significant results when taken individually. Fisher's exact test and likelihood ratio χ^2 tests, in the R environment (R Project for Statistical Computing, <http://www.r-project.org/>), were performed to determine individual and global P -values, respectively. Bonferroni correction was used to avoid inflation of type 1 error, accounting for 12 comparisons resulting from the analysis of four genotype patterns for the whole, the sex-specific, and the phenotype-specific samples. This resulted in a statistical significance threshold of $p < 0.004$ ($0.05/12$).

Population Structure Analysis: We genotyped an additional 348 unrelated African-American subjects with the same Illumina candidate-gene array used above for inclusion in Bayesian cluster analysis to serve as a proxy ancestral population. Based on 113 AIMs, STRUCTURE 2.2 was used to assess population stratification in the case and control subjects by estimating the ancestry proportion for each EA and AA subject. This also established whether the self-reported ethnicity was consistent with the genetically identified ethnicity. Self-identified EA and AA subjects ($N=1,216$, total) were included in each STRUCTURE run. Parameters were set to 100,000 burn-ins followed by 100,000 iterations; the number of genetic clusters, k , was set from 1 to 5 and the best k was inferred by the rate of change in the log probability of data, $\text{LnP}(D)$, between successive k values [Evanno et al., 2005].

RESULTS

Data Mining: Association Analysis for 130 Candidate Genes

Analysis of the 1,350 markers showed that several candidate genes were significantly associated with AFDs (data not shown). *NGFB*, the most strongly associated gene with PAFDs in women, was of particular interest because it showed differential association in PAFD and CAFD subjects and in women and men. After 100,000 permutations based on 1,350 SNPs, one SNP (rs2856813) in *NGFB* was significantly associated with AFDs (empirical $P = 0.005$). However, after stratification by sex, six SNPs in *NGFB* showed significant association with AFDs in women (empirical P s = 0.001–0.03), but none in men. After stratification by AFD type, six markers (including rs2856813) were significantly associated with PAFDs (empirical P s = 0.004–0.04). The observed association for these six

SNPs with PAFDs increased in magnitude when only women were included in the analysis (empirical P s = 0.001–0.02), but no SNP showed significant association with PAFDs in men only. Except for rs2856813, which showed a modest, but significant association with CAFDs in women (empirical P = 0.03), no other markers were associated with CAFDs in either sex (Table III).

Association Analysis for *NGFB*

Single-Marker Association Analysis—In this step, we focused on the *NGFB* gene, which showed the strongest association with PAFDs in women, for the reasons we mentioned in the introduction. We excluded three markers (SNPs 1, 2, and 5) with $MAF < 0.025$ (Table II), leaving 12 SNPs for the association analysis. Considering all of the AFD samples, four markers showed nominally significant association with AFDs (P = 0.004–0.048), and SNP 9 (rs2856813) remained significant after 1,000,000 permutations (empirical P = 0.038). After stratification by sex, seven markers (P 's = 0.0007–0.04), showed stronger significant association with AFDs in women; two of these remained significant after permutations (empirical P 's = 0.008 and 0.018). No markers showed significant association with AFDs in men.

We also examined the associations separately for PAFDs and CAFDs. Six markers displayed stronger nominal association with PAFDs (P 's = 0.004–0.037); these were the same markers previously found in the data mining to be associated with AFDs in women (Table III). After permutation tests, two remained significantly associated with PAFDs (empirical P 's = 0.029 and 0.04). No markers showed significant association with CAFDs.

We also stratified PAFDs and CAFDs by sex. We found that the effects of all six positive markers identified in the PAFD group were attributable to the association in women, which yielded greater statistical significance (P 's = 0.0007–0.01). Three of the SNPs (SNPs 9, 13, and 14) remained significant in the permutation test (empirical P 's = 0.008–0.015). No markers showed association with PAFDs in men. Also, no markers showed significant association with CAFDs in either sex after permutations (Table IV and Figure 2).

Using JMP Genomics and Haploview software yielded similar results for the single marker association analysis.

Haplotype Association Analysis—The LD structure from the present haplotype analysis was consistent with that calculated using information from the HapMap database for this gene region. We observed 3 blocks, which consisted of SNPs 3, 4, and 6 (block 1), SNPs 9 and 10 (block 2), and SNPs 11 and 12 (block 3) (Figure 1). Taking into account the LD structure and the positive markers in a markerwise analysis, SNPs 9, 11, and 12, identified as Cluster 1, were selected for additional haplotype association analysis.

Haplotype association analysis showed that T-G-G in block 1, A-C and G-T in Block 2, and A-G-G, G-A-A in Cluster 1 were nominally significantly associated with AFDs (P s = 0.004–0.03), and A-C and A-G-G retained significance (empirical P s = 0.02 and 0.03, respectively) after 1,000,000 permutations (Table V). The association between haplotypes and AFDs was more extensive and stronger after subgroup and sex stratification, as for single-SNP analysis. Seven haplotypes, including the five listed above, showed greater statistical evidence of association to AFDs in women before multiple testing (P s = 0.0008–0.04) and four of these remained significant after multiple testing was accounted for (empirical P s = 0.005–0.0037). However, none of the haplotypes showed significant association with AFDs in men. In the PAFD samples, before multiple test correction, six haplotypes (P s = 0.0009–0.04) showed significant association with PAFDs, with five remaining significant after permutations (empirical P s = 0.002–0.04). Seven haplotypes

showed significant association with PAFDs in women before ($P_s = 0.001$ – 0.01) and six after permutations (empirical $P_s = 0.006$ – 0.049). There were no haplotype associations in men. In the CAFD samples, only one haplotype showed nominally significant association with CAFDs in women ($P = 0.03$), but this was not significant after permutations (Table V and Figure 2).

Since the CAFD group included 168 subjects with MD and 46 with BP, we also considered the MD and BP subgroups individually. After multiple test correction, no SNP or haplotype showed association with MD or BP (Supplementary Table I and II).

In this study, the controls were younger (mean age = 30.2) than the CAFD cases (mean age = 39.7). As some controls may become affected later in life and thus could be misclassified, we repeated the association analysis excluding control subjects younger than 26 years (resulting mean age for controls, 37.4, $N=259$). Two single markers (rs2856813 and rs4332358) and two haplotypes (A–C and G–C in block 2) remained significantly associated with PAFD in women in the permutation test (empirical $P_s = 0.0497$ – 0.0326) (Supplementary Table III), but no markers or haplotypes showed association with PAFD in men. We did not observe any single marker or haplotype associated with CAFD in men.

Diplotype Analysis—Diplotype association analysis showed strong evidence of association with PAFDs in women. Four genotype patterns (SNPs 9-10, SNPs 11-12, SNPs 9-11-12, and SNPs 9-10-11-12) showed significant association (global $P_s = 0.001$ – 0.02) (Table VIa and Figure 2). After Bonferroni adjustment, the association with SNPs 9-10 remained statistically significant (Table VIa). The specific diplotype GG-TC from the SNP 9-10 pattern showed strongly significant association with PAFDs in women ($OR = 4.07$, $P = 0.000042$) (Table VIb). There was no evidence of association with PAFDs or CAFDs in men.

Population Structure Analysis—According to the distribution of the rate of change in the log probability of data between successive k values [Evanno et al., 2005], as shown in Figure 3, the number of clusters that best fit the data set was two, corresponding to two ancestries: AAs (additional samples were included to set AA reference ancestry for improved clustering accuracy) and EAs (our current study subjects) (Figure 4). Based on inferred ancestry proportions, the admixed samples were allocated into two populations: “genetic” EAs with European ancestry proportion more than 50%, and “genetic” AAs with African ancestry proportion more than 50%. The concordance between the genetic ancestry and the self-reported ancestry are 99.8% (866/868) in EAs and 98.8% (344/348) in AAs. The average European ancestry proportion was 86% in the 868 genetic EA subjects, and 18.7% in the 348 genetic AA subjects. In the EA subjects, the average European ancestry proportion was 86.3% in cases and 86.4% in controls. There were no statistically significant differences in admixture between case and control groups ($t = 0.04$, $df = 866$, $P = 0.97$). Thus, stratification was ruled out as a source of spurious association in our sample sets.

DISCUSSION

We examined the role of variation in *NGFB* in risk of PAFDs and CAFDs. Our findings showed that *NGFB* is strongly associated with PAFDs, but not with CAFDs. The association between *NGFB* and PAFDs was observed only in women.

We excluded population stratification as a source of the observed associations through the analysis of data on 113 SNP AIMs from 868 EAs and 348 AAs. To correct for multiple comparisons in the single-marker and haplotype association analyses, we used the

permutation test with 1,000,000 permutations to estimate empirical *P*-values. For the diplotype association analysis, because we could not identify suitable software to conduct permutation-based analysis, we used the more conservative Bonferroni adjustment. The Bonferroni adjustment is based on the assumption that the tests are independent, so it is conservative because markers in *NGFB* are in linkage disequilibrium. We observed strong evidence of association between *NGFB* and PAFDs in women after stringent correction of multiple testing. Although the positive markers within and near *NGFB* were selected from 1,350 SNPs from the 130-candidate-gene association analysis, we evaluated statistical significance by means of a permutation test based on the full set of 1,350 markers in a data mining step that avoided the accumulation of type I errors. Thus, there is significant evidence of association of *NGFB* with PAFDs in women, shown in single-marker, haplotype, and diplotype analyses.

The association between *NGFB* and PAFDs that we observed supports a role of *NGFB* in the development of PAFDs, consistent with results from animal studies [Aloe et al., 2002; Marais et al., 2008]. *NGFB* has been shown to play a key role in the development and function of the CNS [Levi-Montalcini, 1987]. Withdrawal of *NGFB* can lead to impaired neuronal function and neuronal apoptosis [Davies, 2000], which could contribute to the development of AFDs [Drevets et al., 2008; Phillips et al., 2008]. Further, it has been shown in animal models and humans that stressful life events alter endogenous NGF synthesis and/or utilization, which in humans lead to the development of AFDs [Alleva and Santucci, 2001; Aloe et al., 2002]. When the first parachute jumps of soldiers were used as a model of human stress, NGF levels in peripheral lymphocytes increased 84% in pre-jump and 107% in post-jump sampling [Alleva and Santucci, 2001]. Endogenous release of NGF during exposure to stressors has been shown to remodel damaged tissues, as a compensatory mechanism [Aloe et al., 2002; Marais et al., 2008]. In animal models, treatment with lithium and electroconvulsive stimuli increased NGF concentrations in the frontal cortex, hippocampus, amygdala and limbic forebrain [Angelucci et al., 2003; Hellweg et al., 2002].

Our results also indicated that the role of *NGFB* in PAFDs might be sex specific. A striking sex difference in the prevalence of AFDs can be seen in different countries and cultures, suggesting that there is a biological basis for it [Altemus, 2006]. However, efforts to identify the biological origin of these sex differences have remained largely unsuccessful. We found that variants in *NGFB* showed significant association in women with PAFDs, but not in men. Our finding is similar to the recent observation that variation in *NGFB* confers susceptibility to anxiety-related personality traits in a sex-dependent way [Lang et al., 2008]. Findings from animal studies show that NGF can be regulated by sex hormones and that females produce less NGF than males [Levi-Montalcini, 1987; Levi-Montalcini and Angeletti, 1968; Wright et al., 1987], which may help to explain the sex-dependent effect observed in our study. Castration in males drastically reduces NGF concentrations in brain, spinal cord and salivary gland, whereas exogenous administration of testosterone to either young or adult females or castrated males increases the concentration of NGF in these tissues [Katoh-Semba et al., 1990; Levi-Montalcini and Angeletti, 1968]. In rhesus macaques, the plasma concentration of NGF increased in response to stress only in males [Cirulli et al., 2009]. Animal studies have shown that stress early in pregnancy may produce a significant sex-dependent effect on placental gene expression, which could alter fetal transport of growth factors and nutrients. These alterations could then exert downstream effects resulting in sex differences in stress sensitivity and psychiatric disease risk (Goel et al., 2009). Males had higher concentrations of NGF in peripheral and brain tissues than females, possibly representing a better adaptation to stressful life events. The sex-dependent effects of *NGFB* in PAFDs might help to explain why AFDs are more prevalent in women than in men.

Our findings also suggest that PAFDs and CAFDs may have different underlying mechanisms, since we found no evidence for association between *NGFB* and CAFDs. PAFDs might result in part from inadequate amounts of endogenous NGF associated with *NGFB* variation, hampering neuronal development and remodeling of damaged neurons following stressful events. On the other hand, risk for CAFDs might be regulated by the effects of neurotoxic substances such as drugs of abuse, which might secondarily lead to inadequate *NGFB* expression, an environmental effect that does not depend on the presence of variation in *NGFB per se*. Consistent with this interpretation, recent studies revealed that chronic heroin, cocaine, cannabis, and alcohol abuse reduced serum concentrations of NGFB [Angelucci et al., 2007; Angelucci et al., 2008]. In view of the neurotrophic hypothesis of AFDs and the findings from these studies, reduced concentration of NGFB could increase the risk of developing AFDs.

Limitations of the current study include the fact that we did not differentiate independent from substance-induced AFDs in CAFD samples because of the limited sample and the difficulties in identifying the exact chronological order of onset of AFDs and SD in individuals with both disorders. Although the CAFD group may include some individuals with primary AFDs comorbid with SD, it is unlikely that the inclusion of such individuals in this subgroup confounded the analysis, because such misclassification would be expected to result in an association between *NGFB* and CAFD, which we did not observe. On the other hand, individuals with both independent and substance-induced major depressive episodes (MDEs) had more severe psychopathology than those with either independent or substance-induced MDE only [Niciu et al., 2009]. This provides support for our interpretation that our findings reflect different pathophysiologic effects in PAFDs and CAFDs, with individuals suffering from both types of AFDs having “a double dose” of pathogenic risk and a concomitantly more severe clinical picture. In addition, our findings derived from EAs, and thus they are applicable to this population only. It is necessary that these findings be confirmed in other populations,

Despite these limitations, our study provides a number of possible genetic and pathophysiologic insights into AFDs. In addition to showing that PAFDs and CAFDs, which represent common disorders, might have different genetic backgrounds and pathophysiologic processes, we found that the association of *NGFB* with PAFDs was sex dependent. This could help to explain the higher prevalence of AFDs in women. The findings may also help to explain why PAFDs respond well to medications, which may serve to alleviate genetically influenced symptoms in PAFDs. In contrast, CAFDs appear to respond effectively to abstinence from substances of abuse [Brown and Schuckit, 1988; Nakamura et al., 1983]. If these findings are confirmed in EAs and extended to other populations, it may be possible to use variation in *NGFB* to determine the best treatment for individuals with affective disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

LD Structures of 15 SNPs Spanning the NGFB Gene in the EA Sample Sets. White color represents $D' < 1$ and $\text{LOD} < 2$; blue color represents $D' = 1$ and $\text{LOD} < 2$; shades of pink/red represent $D' < 1$ and $\text{LOD} \geq 2$; bright red represents $D' = 1$ and $\text{LOD} \geq 2$; the empty box represents

FIG. 2.

Single-Marker, Haplotype, and Diplotype Association. SM, single-marker; Hap, Haplotype; Dip, diplotype. A, B, and C: Genetic association between *NGFB* and AFDs, PAFDs, and CAFDs after stratification by AFD type, a, b, and c: Genetic association between *NGFB* and AFDs, PAFDs, and CAFDs after stratification by sex. *P* values showed in the figure were before multiple testing. N=cases+controls

FIG. 3. Probability Estimates for the Number of Clusters. The ordinate shows the Ln probability corresponding to the number of clusters (k) shown on the abscissa.

FIG. 4. Ancestry Structure of Individuals, x axis = individuals; y axis = ancestry proportions, a = EAs, and b = AAs in the x-axis; European ancestry proportions: red, and African ancestry proportions: green.

TABLE I

Characteristics of the European American Samples

		Total		Age		Female		Male	
		N	Mean	SD	N	%	N	%	N
Sample		868	NA		497	57.3%			371
Cases	AFDs	396	NA		222	56.1%			174
	PAFDs	182	NA		109	59.9%			73
	CAFDs	214	39.67	10.98	113	52.8%			101
Controls		472	30.21	11.04	275	58.3%			197
CAFD(N=214, including 46 BP and 168 MD)									
AFD & SD	N	AFD & other PD	N	Diagnostic		Comorbidity		N	
Alcohol	114	ADHD	26	DD		DD&AD		1	
Cocaine	145	Agoraphobia	23			DD		2	
Opitoid	117	ASPD	29	MD with Seasonal Pattern		12		12	
Sedative	37	CD	20			MD&+AD		10	
Stimulant	31	GAD	5			MD&PTSD		5	
Tobacco	156	Gambling	18	MD		167		MD&OCD	
Other drugs	44	OCD	14			MD&social phobia		2	
		Panic disorder	50			MD		148	
		PTSD	54	Total		182		182	
		Social phobia	21						

PAFDs: Primary Affective Disorders;

CAFDs: Affective Disorders Comorbid with Substance Dependence;

NA: Not Available

Anxiety Disorder (AD), Antisocial personality disorder (ASPD), Bipolar Disorder (BP), Conduct disorder (CD), Dysthymic Disorder (DD), Generalized Anxiety Disorder (GAD), Major Depression (MD), Obsessive-Compulsive Disorder (OCD), Post-Traumatic Stress Disorder (PTSD), Psychiatric Disorder (PD)

TABLE II

Characteristics of SNPs in *NGFB*

SNP number	db SNP rs number	SNP type	Chromosome position (bp)	Distance (bp) from previous marker (bp)	Allele	HW P	SNP Call Rate %	MAF
1	rs1146611	Exon	115540798	...	C:T	0.01	100	0.002 *
2	rs1146611	Exon	115541245	447	G:A	1	99.4	0.001 *
3	rs6330	Exon	115541355	110	C:T	0.56	99.1	0.44
4	rs6328	Intron	115541985	630	G:T	0.95	99.8	0.37
5	rs6326*	Intron	115542503	518	G:C	0.01	99.7	0.003 *
6	rs2268793	Intron	115543825	1322	G:A	0.92	100	0.06
7	rs910330	Intron	115547542	3717	C:A	0.32	99.9	0.26
8	rs2239622	Intron	115549751	2209	C:T	0.45	100	0.26
9	rs2856813	Intron	115549961	210	A:G	1	99.9	0.45
10	rs6678788	Intron	115551713	1752	C:T	0.31	99.7	0.26
11	rs4529705	Intron	115563533	11820	G:A	0.78	99.9	0.30
12	rs6537860	Intron	115568386	4853	G:A	0.77	100	0.30
13	rs1077679	Intron	115585089	16703	T:G	0.12	99.9	0.20
14	rs432358	Intron	115587687	2598	C:T	1	99.9	0.28
15	rs3811014	Promot	115594545	6858	A:G	0.37	99.9	0.19

* Markers were excluded from association analyses due to MAF < 0.025.

The Results of Single-Marker Association Analyses of *NGFB* after Permutations Based on the Full Set of Candidate Genes

TABLE III

SNP No.	rs Number	AFD		AFD-F		AFD-M		PAFD		PAFD-F		PAFD-M		CAFD		CAFD-F		CAFD-M	
		Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>	Empirical <i>P</i>
3	rs6330			0.0098		0.043		0.016											
9	rs2856813	0.0046		0.0013		0.007		0.0018									0.03		
10	rs6678788	0.046																	
11	rs4529705			0.034		0.0042		0.0077											
12	rs6537860					0.0058		0.012											
13	rs10776799			0.032		0.0076		0.0024											
14	rs4332358			0.0023		0.033		0.0011											
15	rs3811014			0.023															

Only empirically-significant *P*-values based on the full set of the candidate genes are listed here.

F: Women, M: Men

TABLE IV

Single-Marker Association Analysis

SNP Number	3	4	6	7	8	9	10	11	12	13	14	15		
AFD	All	χ^2	4.01	1.36	0.91	3.72	1.14	8.14	4.17	3.9	3.6	2.67	3.24	1.51
		P	0.045	0.24	0.34	0.05	0.29	0.004	0.04	0.048	0.06	0.1	0.07	0.22
		$Emp\ P$	0.3	0.86	0.96	0.35	0.91	0.038	0.28	0.32	0.37	0.55	0.44	0.83
	Women	χ^2	6.94	1.21	2.79	1.74	0.38	11.43	1.87	4.64	4.22	5.28	9.87	5.17
		P	0.0084	0.27	0.09	0.19	0.54	0.0007	0.17	0.03	0.04	0.02	0.0017	0.02
		$Emp\ P$	0.08	0.91	0.54	0.79	1	0.008	0.76	0.24	0.28	0.17	0.018	0.18
	Men	χ^2	0	0.25	0.26	2.35	0.93	0.25	2.76	0.31	0.31	0.03	0.78	0.55
		P	0.96	0.62	0.61	0.13	0.33	0.61	0.1	0.58	0.58	0.86	0.38	0.46
	ALL	χ^2	4.36	1.74	2	2.32	1.34	7.51	2.96	8.81	8.16	7.57	4.86	0.35
		P	0.037	0.19	0.16	0.13	0.25	0.006	0.09	0.003	0.004	0.0059	0.028	0.56
PAFD		$Emp\ P$	0.27	0.78	0.71	0.63	0.87	0.06	0.5	0.029	0.04	0.05	0.2	1
	Women	χ^2	6.24	1.4	2.09	0.34	0.26	10.4	0.61	7.92	7.14	10.3	11.5	3.36
		P	0.01	0.24	0.15	0.56	0.61	0.001	0.43	0.0049	0.0075	0.0014	0.0007	0.067
		$Emp\ P$	0.12	0.87	0.71	1	1	0.01	0.99	0.05	0.07	0.015	0.008	0.43
	Men	χ^2	0.07	0.43	0.24	2.87	1.43	0.13	3.1	1.47	1.47	0.1	0.43	1.58
		P	0.79	0.51	0.62	0.09	0.23	0.72	0.08	0.23	0.23	0.76	0.51	0.21
	ALL	χ^2	1.39	0.36	0.03	2.56	0.35	3.64	2.57	0.12	0.11	0	0.61	1.85
		P	0.24	0.55	0.86	0.11	0.55	0.06	0.11	0.73	0.74	1	0.43	0.17
		$Emp\ P$	0.93	1	1	0.73	1	0.6	0.73	1	1	1	1	0.84
	Women	χ^2	3	0.36	1.74	2.36	0.24	4.94	2	0.46	0.43	0.27	3.01	3.55
CAFD		P	0.08	0.55	0.19	0.12	0.63	0.03	0.16	0.5	0.51	0.6	0.08	0.06
		$Emp\ P$	0.48	1	0.77	0.64	1	0.2	0.71	0.99	1	1	0.48	0.39
	Men	χ^2	0.02	0.04	1.45	0.81	0.2	0.2	1.07	0.03	0.03	0.26	0.58	0.01
		P	0.88	0.85	0.23	0.37	0.65	0.65	0.3	0.86	0.86	0.61	0.45	0.94

Emp P: Empirical *P*-value

TABLE V

Haplotype Association Analysis

Haplotypes	Block 1			Block 2			Block 3			Cluster 1					
	T-G-G	C-T-G	C-G-G	C-G-A	A-C	G-T	G-C	G-G	A-A	A-G-G	G-A-A	G-G-G	A-A-A		
AFD	All	Freq.	0.44	0.37	0.13	0.06	0.55	0.26	0.19	0.7	0.3	0.53	0.28	0.18	0.02
		χ^2	4.55	1.04	0.7	0.91	8.26	4.49	1.61	3.73	3.73	6.37	5.61	0.98	1.66
		P	0.03	0.31	0.4	0.34	0.004	0.03	0.2	0.05	0.05	0.01	0.02	0.32	0.2
		Empirical P	0.17	0.91	0.96	0.93	0.02	0.17	0.73	0.26	0.26	0.03	0.05	0.69	0.48
	Women	Freq.	0.43	0.37	0.13	0.07	0.53	0.29	0.19	0.69	0.31	0.51	0.29	0.18	0.02
		χ^2	7.07	1.11	1.34	2.79	11.19	2.04	7.37	4.53	4.22	9.12	6.46	1.87	2.19
		P	0.01	0.29	0.25	0.09	0.0008	0.15	0.007	0.03	0.04	0.0025	0.01	0.17	0.13
		Empirical P	0.05	0.91	0.77	0.42	0.005	0.58	0.037	0.19	0.21	0.005	0.02	0.3	0.39
	Men	Freq.	0.44	0.38	0.12	0.06	0.57	0.23	0.2	0.72	0.28	0.55	0.25	0.17	0.02
		χ^2	0.04	0.11	0	0.26	0.25	2.78	1.25	0.31	0.31	0.18	0.43	0.01	0.08
	P	0.84	0.74	0.95	0.61	0.61	0.1	0.26	0.58	0.58	0.67	0.51	0.92	0.78	
PAFD	All	Freq.	0.44	0.37	0.12	0.06	0.56	0.25	0.19	0.7	0.3	0.53	0.28	0.17	0.02
		χ^2	4.36	1.39	0.12	2.08	7.6	3.18	2.29	8.52	8.52	6.29	10.98	0.05	1.08
		P	0.04	0.24	0.73	0.15	0.0058	0.07	0.13	0.004	0.004	0.01	0.0009	0.82	0.3
		Empirical P	0.22	0.79	1	0.61	0.04	0.38	0.56	0.027	0.027	0.037	0.002	0.99	0.68
	Women	Freq.	0.44	0.37	0.13	0.06	0.54	0.28	0.18	0.69	0.31	0.52	0.29	0.17	0.02
		χ^2	6.11	1.3	0.95	2.09	10.2	0.7	10.4	7.8	7.14	8.94	9.58	0.29	1.31
		P	0.01	0.25	0.33	0.15	0.001	0.4	0.001	0.005	0.0075	0.0028	0.002	0.59	0.25
		Empirical P	0.08	0.89	0.94	0.76	0.0096	0.97	0.009	0.035	0.049	0.0045	0.006	0.65	1
	Men	Freq.	0.44	0.38	0.11	0.07	0.57	0.22	0.2	0.72	0.28	0.55	0.26	0.16	0.02
		χ^2	0.17	0.26	0.58	0.24	0.13	3.1	1.91	1.47	1.47	0.08	1.74	1.21	0.07
	P	0.68	0.61	0.45	0.62	0.72	0.08	0.17	0.23	0.23	0.78	0.19	0.27	0.8	
CAFD	All	Freq.	0.45	0.37	0.13	0.06	0.56	0.25	0.19	0.72	0.28	0.54	0.26	0.18	0.02
		χ^2	1.75	0.3	1.08	0.04	3.71	2.78	0.35	0.11	0.11	2.6	0.43	2.9	0.91
		P	0.19	0.59	0.3	0.84	0.05	0.1	0.55	0.74	0.74	0.11	0.51	0.09	0.34
	Women	Freq.	0.45	0.36	0.13	0.06	0.55	0.28	0.17	0.71	0.29	0.53	0.27	0.18	0.02

Haplotypes	Block 1			Block 2			Block 3			Cluster 1			
	T-G-G	C-T-G	C-G-G	C-G-A	A-C	G-T	G-C	G-G	A-A	A-G-G	G-A-A	G-G-G	A-A-A
χ^2	3.23	0.31	0.85	1.74	4.82	2.14	1.51	0.43	0.43	3.52	1.08	2.76	1.2
P	0.07	0.58	0.36	0.19	0.03	0.14	0.22	0.51	0.51	0.06	0.3	0.1	0.27
Empirical P	0.32	1	0.93	0.76	0.15	0.55	0.81	0.99	0.99				
Freq.	0.44	0.37	0.12	0.06	0.57	0.22	0.21	0.73	0.27	0.55	0.25	0.18	0.02
χ^2	0	0.01	0.25	1.45	0.2	1.09	0.25	0.03	0.03	0.16	0.01	0.5	0.03
P	0.97	0.94	0.61	0.23	0.65	0.3	0.62	0.86	0.86	0.69	0.91	0.48	0.85

TABLE VI

Diplotype Association Analysis

a. Global diplotype association									
AFD			PAFD			CAFD			
Genotype Patterns	χ^2	df	Global P	χ^2	df	Global P	χ^2	df	Global P
All									
SNP 9-10	13.34	6	0.04	12.79	6	0.047	7.49	6	0.28
SNP 11-12	5.82	3	0.12	11.6	3	0.009	6.57	2	0.04
SNP 9-11-12	14.66	8	0.07	15.94	8	0.04	16.08	7	0.02
SNP 9-10-11-12	27.88	17	0.046	30	17	0.026	22.05	16	0.14
Women									
SNP 9-10*	17.95	6	0.006	21.78	6	0.001	6.96	6	0.32
SNP 11-12	6.91	3	0.08	9.7	3	0.02	5.69	2	0.058
SNP 9-11-12	20.86	8	0.008	20.06	8	0.01	16.27	7	0.02
SNP 9-10-11-12	33	17	0.01	35.36	17	0.006	23.25	16	0.11
Men									
SNP 9-10	6.22	5	0.29	6.32	5	0.28	3.39	5	0.64
SNP 11-12	0.31	2	0.86	2.64	2	0.27	1.61	2	0.45
SNP 9-11-12	5.03	7	0.66	11.3	7	0.13	4.64	7	0.7
SNP 9-10-11-12	16.52	15	0.35	19.68	14	0.14	15.49	15	0.42
b. Association between Specific Diploypes from SNP 9-10 Genotype Pattern and PAFD in Women									
Diplotype	Pooled Freq%	Case	Freq%	Control	Freq%	P-value	Odds Ratio	95%CI	
AA-CC	31.2	28	25.9	91	33.3	0.16	0.70	0.43–1.15	
AG-TC	26.	21	19.4	80	29.3	0.045	0.58	0.34–0.99	
AG-CC	19.2	20	18.5	53	19.4	0.84	0.94	0.54–1.66	
GG-TC*	10.5	23	21.3	17	6.2	4.2E-05	4.07	2.09–7.93	
GG-TT	8.9	10	9.3	24	9.0	0.89	1.06	0.49–2.26	
GG-CC	3.4	6	5.6	7	2.7	0.17	2.23	0.76–6.54	
AG-TT	0.3	0	0	1	0.4	NA			
Total	100	108	100	273	100				

Significant Bonferroni $P < 0.004$.

* Significant diplotype