

ORIGINAL ARTICLE

The α -endomannosidase gene (*MANEA*) is associated with panic disorder and social anxiety disorderKP Jensen^{1,2}, MB Stein³, HR Kranzler⁴, BZ Yang^{1,2}, LA Farrer⁵ and J Gelernter^{1,2,6}

Unbiased genome-wide approaches can provide novel insights into the biological pathways that are important for human behavior and psychiatric disorder risk. The association of α -endomannosidase gene (*MANEA*) variants and cocaine-induced paranoia (CIP) was initially described in a study that used a whole-genome approach. Behavioral effects have been reported for other mannosidase genes, but *MANEA* function in humans and the clinical potential of the previous findings remain unclear. We hypothesized that *MANEA* would be associated with psychiatric phenotypes unrelated to cocaine use. We used a multi-stage association study approach starting with four psychiatric disorders to show an association between a *MANEA* single-nucleotide polymorphism (SNP; rs1133503) and anxiety disorders. In the first study of 2073 European American (EA) and 2459 African American subjects mostly with comorbid drug or alcohol dependence, we observed an association in EAs of rs1133503 with panic disorder (PD) (191 PD cases, odds ratio (OR) = 1.7 (95% confidence interval (CI): 1.22–2.41), $P = 0.002$). We replicated this finding in an independent sample of 142 PD cases (OR = 1.53 (95% CI: 1.00–2.31), $P = 0.043$) and extended it in an independent sample of 131 generalized social anxiety disorder cases (OR = 2.15 (95% CI: 1.27–3.64), $P = 0.004$). *MANEA* alleles and genotypes were also associated with gene expression differences in whole blood cells. Using publically available data, we observed a consistent effect on expression in brain tissue. We conclude that pathways involving α -endomannosidase warrant further investigation in relation to anxiety disorders.

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INTRODUCTION

Understanding the genetic contributions to psychiatric disorders could provide crucial insight into the mechanisms underlying these disorders, which as a group have a lifetime prevalence in US adults of 46.4%.¹ There is moderate heritability for many psychiatric disorders, for example, for panic disorder (PD) it is estimated to be as high as 0.43, and for generalized anxiety disorder as high as 0.32.² Further, the genetic risk can be shared between different psychiatric disorders. Hettema *et al.*³ reported that the genetic risk for generalized anxiety disorder, PD and agoraphobia was similar and that this genetic risk partly overlapped with the risk for social phobia. Shared genetic effects have recently been described for five major psychiatric disorders: schizophrenia, bipolar disorder, autism, major depressive disorder (MDD) and attention deficit-hyperactive disorder based on a large mega-analysis of GWAS data.⁴ The mechanisms that influence the development of these pathological conditions are complex, involving multiple genes and gene regulatory networks and their interactions with the environment. Thus, identifying genetic risk alleles has been challenging. Other factors increasing the difficulty of identifying associations between specific genes and individual disorders are a high rate of comorbidity among disorders, with both common and disorder-specific genetic risk, and environmental factors that may influence how a genetic risk allele common to multiple disorders confers risk for a specific disorder.³

This study, which examines the association of a *MANEA* 3'UTR SNP, rs1133503, with psychiatric disorder risk, is based on previous reports of the behavioral effects of the α -endomannosidase gene (*MANEA*) and related pathways. Two studies have investigated the functions and phenotypes associated with *MANEA* in humans. Yu *et al.*⁵ used a low-density whole-genome association study to investigate the genetic basis for substance dependence phenotypes in European American (EA) and African American (AA) families. They observed that the minor allele of a SNP in the *MANEA* gene (rs1133503*C) was less frequent in subjects affected with cocaine-induced paranoia (CIP), a transient effect of cocaine observed in 60–80% of cocaine-dependent individuals. Farrer *et al.*⁶ investigated the association between CIP and a more comprehensive set of *MANEA* gene variants. Again the rs1133503 SNP was robustly associated with CIP in the AA and EA family-based studies, but these effects were less robust in AA and EA case-control populations. In the case-control analysis, rs1133503 was associated with CIP in AAs but not EAs.⁶

Our knowledge of *MANEA* remains limited, but certain mannosidase genes have been investigated extensively because of their involvement in alpha and beta mannosidosis, rare diseases characterized by varying degrees of developmental and behavioral abnormalities and cognitive impairment.^{7–10} Twenty-five percent of alpha-mannosidosis cases have psychiatric manifestations that include hallucinations, depression and/or severe anxiety

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Table 1. Demographic information and comorbid diagnoses for anxiety case and control samples

	Sample 1 panic disorder (EA)		Sample 2 panic disorder (EA)		Sample 3 GSAD (EA)		Panic disorder (AA)	
	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases
Age (mean)	38.6	38.1	43.7	38.7	19	39.6	41.8	41.1
Male (%)	57.7	39.8	41.8	38	33.7	70.7	54.8	40.3
Alcohol dependent (%)	55.1	79.8	0	24.4	0	0	58.8	80.7
Cocaine dependent (%)	54	78.7	0	18.9	0	0	73.2	81.7
Opioid dependent (%)	43.6	76.2	0	25.6	0	0	20.1	33.3
Cocaine-induced paranoia (%)	37.7	57.6	0	0	0	0	50.5	66.1
No dependence (%)	27.4	2.6	100	43.3	0	0	17.4	6.8
Total	1882	191	550	142	202	131	2398	61

Abbreviations: AA, African American; EA, European American; GSAD, generalized social anxiety disorder.

that sometimes precede other neurological abnormalities.^{11,12} An acute form of mannosidosis can occur in livestock that ingest large quantities of swainsonine-containing plants called 'locoweeds.' Swainsonine is a naturally occurring plant alkaloid that inhibits the golgi alpha-mannosidase II enzyme, causing unprocessed manose to accumulate in intracellular vacuoles.^{13,14} Affected animals begin to display symptoms that include aggression and hyperactivity, which is often referred to as 'locoism.'

Because psychiatric risk genes can have pleiotropic effects,⁴ we hypothesized that *MANEA* gene variants could contribute to the risk for psychiatric disorders beyond those directly related to cocaine use. To understand better the functions of *MANEA* in humans, we used a multi-stage association study to investigate the effect of a *MANEA* SNP (rs1133503) on risk for psychiatric disorders, followed by a functional analysis of *MANEA* SNPs and their potential effect on gene expression. We limited our association analysis to rs1133503 because it tags the two major common haplotypes that span the gene, and, in two previous family-based studies, this particular SNP was associated with CIP in EAs and AAs.^{5,6} Both previous studies reported that individuals carrying the rs1133503 minor allele (rs1133503*C) were less likely to be affected with CIP. First, we explored the association of rs1133503 with four psychiatric disorders (PD, MDD, antisocial personality disorder (ASPD) and post-traumatic stress disorder (PTSD)) prevalent in subjects recruited for genetic studies of cocaine, alcohol or opioid dependence. A second set of association analyses was initiated to provide additional support for an association between *MANEA* and pathological anxiety in independent samples. Then, to investigate the functional effects of *MANEA* gene variants, we examined the association between *MANEA* variants and *MANEA* mRNA levels in human blood cells and in brain cells, using publically available data.¹⁵ The effects of *MANEA* variants on post-transcriptional and transcriptional gene regulatory mechanisms were tested *in vitro*.

MATERIALS AND METHODS

Subjects

Subjects in samples 1 and 2 were recruited for studies of the genetics of drug (cocaine or opioid) and alcohol dependence or anxiety disorders. The subjects were interviewed using the polydiagnostic Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA)^{16,17} to obtain *DSM-IV* diagnoses for all major psychiatric disorders and the additional variables used in the analysis. *DSM-IV* diagnoses require that the comorbid psychiatric diagnoses are not the result of substance dependence or other substance use. These samples include subjects analyzed in previous genetic studies of CIP.^{5,6} Fifty-two PD cases from sample 2 were recruited from primary care settings for the Coordinated Anxiety Learning and Management (CALM) study.¹⁸ Sample 3 included 131 EA subjects

diagnosed with generalized social anxiety disorder (GSAD) and 202 EA controls who were volunteers recruited from a college population (University of California San Diego, La Jolla, CA, USA) and who denied symptoms consistent with a diagnosis of social anxiety disorder. A diagnosis of GSAD was made using a semi-structured diagnostic interview (MINI)¹⁹ by experienced clinical interviewers. The institutional review board at each participating site approved the studies, and all subjects provided written informed consent to participate. Recruitment sites are shown in Supplementary Table S1.

The National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism issued Certificates of Confidentiality to protect study participants. The frequencies of the four disorders in our primary analysis (Table 2) were PD-9.1, ASPD-12.4, MDD-15.4 and PTSD-13.5% in the EA sample, and PD-2.5, ASPD-11.7, MDD-11.39 and PTSD-13.3% in the AA sample. Demographic information and major comorbid diagnosis for anxiety cases and controls are shown in Table 1. More details concerning the subjects are available in the Supplementary Information.

Genotyping

The rs1133503 SNP was genotyped with a 2 µl TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) as described in the Supplementary Information or with the Infinium HumanCore BeadChip (Illumina, San Diego, CA, USA) that included custom content.

Gene expression and cell culture experiments

At Yale, whole blood was collected from subjects with PAXgene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland). To extract RNA, the blood samples were first centrifuged for 10 min at 2000g. The cell pellet was washed with 4 ml of H₂O and centrifuged again for 10 min at 2000g. RNA was extracted from the cell pellet with 1 ml of Trizol (Invitrogen, Carlsbad, CA, USA). RNA was reverse transcribed using the ABI High Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's specifications. The allelic imbalance assays were performed using the TaqMan genotyping reaction described above but in 10 µl with either 5 ng of genomic DNA or a twentieth of the cDNA reaction. Lymphoblastoid cells were cultured at 37 °C in RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin. After actinomycin D treatment (1 µg ml⁻¹), RNA was extracted with Trizol, reverse transcribed with the ABI High Capacity cDNA Archive Kit and amplified in a 10 µl PCR reaction containing 250 nm forward and 250 nm reverse primer, 0.1x SYBR Green I (Molecular Probes, Eugene, OR, USA), 0.1 µg bovine serum albumin (New England Biolabs, Ipswich, MA, USA) and 1x TaqMan Universal Master Mix (Applied Biosystems). A twentieth of each cDNA reaction was used as a template for *MANEA* and *HPRT* detection, and a hundredth of the cDNA reaction was used for 18s detection. Primer sequences are available in Supplementary Information. RT-PCR reactions were performed in triplicate and the relative level of gene expression determined with the $\Delta\Delta C_t$ method. Brain mRNA expression was analyzed with the help of the web site <http://braincloud.jhmi.edu> using genotype information from dbGaP (phs000417.v1.p1).¹⁵

Statistical analysis

Subjects were grouped as EA or AA based on self report. Ancestries were verified with *Structure*²⁰ software or principal component (PC) analysis,²¹ and subjects with discordant self-reports were excluded from the analysis. Self-reported ancestry was confirmed using *Structure*²⁰ and a panel of EA and AA ancestry informative STR and SNP markers²² (with additional informative SNPs, rs1540771 (6p23.5), rs1805007 (*MC1R*), rs12896399 (*SLC24A4*) and rs1426654 (*SLC24A5*)) or a panel of 96 SNPs that differentiate African, Asian, European and Mexican populations. A subset of ancestries was confirmed based on the first two PCs²¹ of a GWAS study of substance dependence^{23–25} or with ancestry informative SNPs included on the Infinium HumanCore BeadChip to distinguish European from African populations. Because multiple methods were used to confirm self-reported ancestry, the ancestry proportion scores (or PCs) were not used as covariates in the regression analysis; however, these methods confirmed that the EA and AA groups were reasonably homogenous genetically. Hardy–Weinberg equilibrium was assessed with Pearson's Chi-squared test ($P > 0.01$ for all case and control groups). Data analysis was performed using JMP 9.0.0 software (Cary, NC, USA), GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and R version 2.15.2 and the SNPpass (v1.8–5) package,²⁶ as described in the text.

RESULTS

MANEA gene variation is associated with anxiety disorders in EAs. We focused the first stage of our association analysis on comorbid psychiatric disorders that were prevalent in our EA and AA samples, which were recruited for genetic studies of alcohol and drug dependence. These disorders were ASPD, MDD, PTSD and PD. The significance level was 0.0125 to correct for testing four phenotypes. *MANEA* genotype was associated with PD risk in EAs only (Table 2). PD risk was elevated for CC (OR = 1.61 (95% CI: 1.01–2.54) $P = 0.046$) and CT (OR = 1.73 (95% CI: 1.22–2.49), $P = 0.002$) relative to TT genotype. Because of the low number of minor allele homozygotes ($n = 35$) in the PD group, we also considered an analysis with minor allele carriers grouped (CC+CT versus TT). In a regression analysis that adjusted for the potential confounders, CIP, sex and age, PD risk was significantly elevated for individuals who carried the C-allele (OR = 1.70 (95% CI: 1.22–2.41), $P = 0.002$; sample 1, Table 3). In an analysis that controlled for comorbid drug (opioid, cocaine or alcohol) dependence, sex and age, the magnitude of the effect was similar, carriers of the C-allele were at increased risk for PD (OR = 1.6, $P = 0.006$). Similarly, controlling for recruitment center (Supplementary Table S1) had negligible effect (OR = 1.69, $P = 0.002$). In the AA sample, no disorder was significantly associated with *MANEA* genotype. Because the prevalence of PD was much lower in the AA sample (< 3%), the power was much lower.

We investigated this effect in an independent EA sample of PD cases ($N = 142$) and controls subjects that had no major psychiatric disorder ($N = 550$). The majority of the PD cases (79%) were recruited specifically for anxiety disorder studies, and CIP-positive PD cases were excluded from this analysis. The effect of carrying the minor allele was consistent with the primary sample, though of a slightly smaller magnitude (sample 2, Table 3). When samples 1 and 2 were combined in a regression analysis that adjusted for sex and CIP, the effect of carrying the C-allele on PD risk was more robustly significant (OR = 1.65 (95% CI: 1.27–2.14), $P = 1.2e-04$).

To investigate further a possible association between *MANEA* and an anxiety phenotype, we studied another independent sample. Because the anxiety phenotype in the second sample was not identical to that in the discovery sample, this was not intended to be a replication *per se*. This sample included 131 EA subjects diagnosed with GSAD and 202 EA controls who were volunteers recruited from a college population and who denied symptoms consistent with a diagnosis of social anxiety disorder. We examined the C-allele (CC+CT genotypes) as a predictor of GSAD diagnosis in a logistic regression model with sex included as

Table 2. The rs1133503 genotype distribution for European American and African American subjects with panic disorder, major depressive disorder, post-traumatic stress disorder and antisocial personality disorder

Group	Genotype count (frequency)			P-value ^a
	TT	CT	CC	
<i>European American</i>				
Non-PD	694 (36.9%)	867 (46.1%)	321 (17.1%)	0.007
PD	49 (25.7%)	107 (56.0%)	35 (18.3%)	
Non-MDD	619 (36.1%)	800 (46.7%)	296 (17.3%)	
MDD	109 (34.9%)	150 (48.1%)	53 (17.0%)	
Non-PTSD	636 (35.4%)	858 (47.8%)	301 (16.8%)	0.1
PTSD	108 (38.4%)	119 (42.4%)	54 (19.2%)	
Non-ASPD	643 (35.8%)	837 (46.6%)	316 (17.6%)	0.68
ASPD	91 (35.7%)	126 (49.4%)	38 (14.9%)	
<i>African American</i>				
Non-PD	1007 (42.0%)	1036 (43.2%)	355 (14.8%)	0.49
PD	28 (45.9%)	27 (44.3%)	6 (9.84%)	
Non-MDD	924 (42.9%)	923 (42.8%)	308 (14.3%)	
MDD	106 (38.3%)	125 (45.1%)	46 (16.6%)	
Non-PTSD	910 (42.7%)	918 (43.1%)	302 (14.2%)	0.18
PTSD	128 (39.0%)	142 (43.3%)	58 (17.7%)	
Non-ASPD	913 (42.4%)	924 (42.9%)	319 (14.8%)	0.84
ASPD	116 (40.7%)	128 (44.9%)	41 (14.4%)	

Abbreviations: ASPD, antisocial personality disorder; MDD, major depressive disorder; PD, panic disorder; PTSD, post-traumatic stress disorder.
^aCodominant regression model adjusted for sex, age and cocaine-induced paranoia.

a covariate. Similar to what we observed in the first-stage analysis, in the second data set, the risk of GSAD was higher for individuals with the C-allele (CC+CT) than those with the TT genotype (sample 3, Table 3).

On the basis of findings of Hettema *et al.*,³ who found similar genetic risk for generalized anxiety disorder, PD and agoraphobia, which partly overlapped with the risk for social phobia, we re-examined the subjects from our primary and secondary samples that were assessed by identical methods, for an association between *MANEA* genotype and a diagnosis count of the four anxiety disorders (generalized anxiety disorder, PD, agoraphobia and social phobia). The C-carrier genotype was associated with the number of diagnoses (Cochran-Armitage trend test, $Z = 2.68$, $P = 0.007$). As the number of diagnoses per subject increased from 0 to 1, 2, 3 and 4, the C-carrier frequency increased from 63.96% ($N = 2220$) to 68.54% ($N = 302$), 74.02% ($N = 127$), 65.38% ($N = 52$) and 90.91% ($N = 11$), respectively. This was not significant in the AA population, which had fewer subjects with multiple diagnoses (0, $N = 2236$; 1, $N = 163$; 2, $N = 26$; 3, $N = 5$).

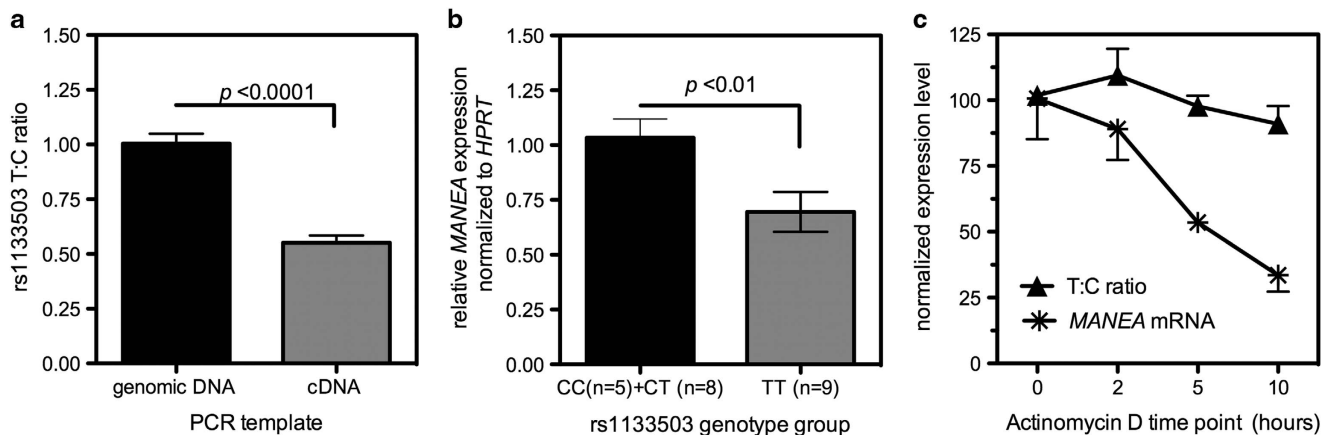
The anxiety disorder risk variant is associated with *MANEA* gene expression differences in blood and brain tissue

The association of rs1133503 with anxiety disorders reported here and with cocaine use behaviors reported previously by Yu *et al.* and Farrer *et al.*, with opposite risk alleles, suggested that the rs1133503 polymorphism might have a direct effect or be in LD with a variant (or variants) that affects *MANEA* function. To test for gene expression differences, we used the anxiety-associated SNP (rs1133503) as a marker of the level of mRNA expressed from each *MANEA* allele in the blood cells of six heterozygous subjects. As shown in Figure 1a, the normalized T:C ratio in cDNA was < 1, suggesting that there was less T-allele mRNA than C-allele mRNA.

Table 3. *MANEA* rs1133503 genotype is associated with panic disorder and generalized social anxiety disorder diagnosis in different European American samples

Group		Genotype count (frequency)			Odds ratio (95% CI) ^a	P-value
		TT	CT	CC		
Sample 1 - panic disorder	Cases	49 (25.7%)	107 (56.0%)	35 (18.3%)	1.7 (1.21–2.40) ^b	0.002
	Controls	694 (36.9%)	867 (46.1%)	321 (17.1%)		
Sample 2 - panic disorder	Cases	36 (25.4%)	74 (52.1%)	32 (22.5%)	1.53 (1.01–2.33) ^c	0.043
	Controls	188 (34.2%)	268 (48.7%)	94 (17.1%)		
Sample 3 - GSAD	Cases	28 (21.4%)	87 (66.4%)	16 (12.2%)	2.15 (1.27–3.64) ^d	0.004
	Controls	74 (36.6%)	96 (47.5%)	32 (15.8%)		

Abbreviation: GSAD, generalized social anxiety disorder.

^aRisk associated with carrying the C-allele of rs1133503.^bAdjusted for sex, age and cocaine-induced paranoia.^cAdjusted for sex and age.^dAdjusted for sex.**Figure 1.** rs1133503 is associated with the level of *MANEA* mRNA in human blood cells. (a) The level of mRNA from the *MANEA* T-allele relative to the C-allele in blood cell RNA from rs1133503 heterozygotes ($N = 6$). Each subject's genomic DNA was amplified in parallel as a reference for 1:1 allele ratio (T:C). (b) The relative level of *MANEA* mRNA in subject blood cells for different rs1133503 genotype groups normalized to *HPRT*. TT subjects (0.696 ± 0.091) compared with subjects that carry the C-allele (1.034 ± 0.086), $P = 0.008$ (one-tailed *T*-test). (c) The T:C ratio in mRNA plotted along with the change in *MANEA* mRNA levels after actinomycin D treatment. In this graph, the T:C ratio at time zero was normalized to 100%. Error bars indicate \pm s.e.m.

Using the genomic DNA as a reference, we observed 45% less T-allele mRNA relative to C-allele mRNA in heterozygous subjects' blood cells ($P < 0.0001$).

We next tested whether the level of *MANEA* mRNA differed between individuals as a function of rs1133503 genotype. Having shown that in heterozygous subjects the T-allele was expressed at lower levels than the C-allele, we hypothesized that the TT genotype would be associated with lower levels of *MANEA* mRNA than C-allele genotypes. To test this hypothesis, we used blood cell RNA that was available from 22 EA subjects (CC=5, CT=8, TT=9). ANOVA showed significant differences in the level of *MANEA* mRNA in relation to each genotype group ($F(2,19) = 4.02$, $P = 0.035$). As shown in Figure 1b, individuals homozygous for the T-allele expressed less *MANEA* mRNA than individuals with CT and CC genotypes, which we grouped in this analysis because of the limited number of CC subjects (TT = 0.696 ± 0.091 versus CC + CT = 1.034 ± 0.086 , $P = 0.008$). The expression difference between the CT and TT group was robust (CT = 1.10 ± 0.122 , $P = 0.008$). There were fewer CC subjects ($n = 5$) and as a group *MANEA* mRNA levels were only modestly different compared with the TT genotype group (CC = 0.929 ± 0.105 , $P = 0.068$), but this difference

was consistent with our previous observation that the relative level of *MANEA* mRNA from the T-allele genotype group was lower. *MANEA* mRNA levels did not differ between CC and CT genotype groups. We used publicly available array-based gene expression data (<http://braincloud.jhmi.edu>¹⁵) to test for a similar effect in brain tissue. *MANEA* rs6940020 was directly genotyped in this sample and it served as a good proxy SNP for rs1133503, as the two are in almost complete LD ($r^2 = 1.0$ (AA) and 0.97 (EA)²⁷). In fetal brain tissue ($n = 37$), the minor allele of rs6940020 was associated with increased *MANEA* mRNA expression ($P = 0.014$) in a general linear model adjusted for race and RNA integrity number (RIN). No effect was observed in adult tissue samples.

To gain additional insight into the biological effects that might be associated with the anxiety risk variant, we investigated whether rs1133503 was associated with a difference in mRNA stability. We cultured lymphoblastoid cell lines from three heterozygous individuals in the presence of a transcriptional inhibitor, actinomycin D. Under normal growth conditions, the T:C ratio in mRNA from the three lymphoblastoid cells was similar to what we observed in whole blood cells, that is, less T mRNA than

C mRNA ($T:C = 0.59 \pm 0.012$). We extracted RNA from the cells at four time points following the addition of actinomycin D (0, 2, 5 and 10 h) and observed no change in the ratio of mRNA from each rs1133503 allele. Evidence that the total amount of *MANEA* mRNA was decreasing after actinomycin D is shown in Figure 1c, where relative to 18s ribosomal RNA, an RNA molecule that is highly stable, the level of *MANEA* mRNA is reduced to 89%, 54% and 34% at 2, 5 and 10 h, respectively, after the addition of actinomycin D. These observations argue that a component of the allelic expression differences might involve changes in the transcription or co-transcriptional processing of each allele.

DISCUSSION

We obtained evidence for association between *MANEA* variation and anxiety disorder risk in independent populations for frequently co-occurring diagnoses that have a common genetic architecture. The effects of *MANEA* on anxiety disorder risk may involve allelic differences in gene expression that we observed in two tissue types: blood and the brain. Although the precise mechanisms underlying the allelic effects on gene expression are not known, they could be mediated by allele-specific differences in transcriptional activity mediated, partly, by allelic-specific differences in the function of SNPs at regulatory regions near the transcription start site. A more detailed examination of the SNPs in this region may be warranted to determine whether they affect the level of *MANEA* expression and anxiety disorder risk. A SNP in this region of *MANEA*, rs7755854, was recently reported to have genome-wide significant effects on DNase1 sensitivity.²⁸ The DNase1 sensitivity sequencing technique used by Degner *et al.*²⁸ uses SNP information to identify allelic alterations in regional chromatin structure and protein binding. Rs7755854 is in high LD with the anxiety-associated SNP studied here, based on 1000 Genome data (CEU, $d' = 0.93$, $r^2 = 0.84$ and YRI, $d' = 1$, $r^2 = 0.82$). Specific neural cell types and brain regions should be examined specifically for similar effects on gene expression. For example, although we observed no effect on mRNA stability, the effects of *MANEA* variants on cell-type-specific effects of regulatory factors, such as microRNA or RNA binding proteins, could be evident in other tissues.^{29,30} Understanding the effects of all of these variants may be challenging because the effects on gene expression can be largely context dependent.

The potential for variation in *MANEA* to contribute to risk of psychiatric phenotypes was initially evidenced by a study that used a whole-genome approach, a method unbiased by prior hypotheses on the mechanisms of disease risk. Genome-wide methods can provide unexpected insights into disease risk, and it can be difficult to integrate such findings in ways that might yield a clinical benefit. Our findings reinforce these previous studies, which identify variation in *MANEA* as an important contributor to psychiatric phenotypes, but our knowledge of *MANEA* remains very limited. How variation in this gene contributes to psychiatric phenotypes in general and anxiety disorders specifically is unknown. Some limited insight may be gleaned from studies on the relationship between other mannosidase genes and cognitive phenotypes. For example, rare missense mutations in mannosidase, alpha, class 2B (*MAN2B1*) and mannosidase, beta A, lysosomal (*MANBA*) cause genetic forms of alpha and beta mannosidosis, respectively, rare diseases characterized by varying degrees of developmental and behavioral abnormalities and cognitive impairment.^{7,8} Also, two recent studies have described rare mutations in the *MAN1B1* gene that cause intellectual disability and cognitive disorders.^{9,10} Mannosidosis caused by mutations in mannosidase genes has also been described in cattle, cats and guinea pigs. However, more research is needed on *MANEA* specifically to understand its relationship with pathological anxiety. Studies that investigate potential behavioral effects of

newly developed endomannosidase inhibitors³¹ may be informative. It is unclear why anxiety traits and CIP have opposite risk alleles. Perhaps these two alleles are associated with high versus low *MANEA* activity and, at high or low extremes, *MANEA* could affect cellular function and disease risk differently. Alternatively, subjects with the anxiety risk genotype may be less likely to consume large amounts of cocaine because they prefer to avoid circumstances likely to trigger heightened anxiety. Some physiological effects of acute cocaine consumption, for example, increased heart rate, mimic anxiety symptoms or can be anxiety provoking in some circumstances. High levels of cocaine consumption are associated with increased risk of CIP,^{32,33} and some anxiety-prone individuals might limit cocaine consumption to a level that is below a threshold to develop CIP. If true, the behavior of some PD subjects might protect them, to some extent, from developing CIP, but it might also mask the effects of *MANEA* genotype on CIP risk. In an analysis of EA cocaine-dependent subjects without PD, rs1133503 genotype was associated with CIP (non-CIP $N = 315$ versus CIP $N = 666$; Cochran–Armitage Trend test, $Z = -2.16$, $P = 0.031$) with the T-allele conferring CIP risk. The effect of genotype was not significant when PD subjects were included in the analysis (non-CIP $N = 360$ versus CIP $N = 781$; $Z = -1.93$, $P = 0.054$). These potentially opposing effects of *MANEA* should be considered in future analyses of these phenotypes.

Our study has limitations. The initial population we studied was enriched with substance-dependent subjects, and PD, MDD, ASPD and PTSD were analyzed as secondary phenotypes. As such, the case groups were comorbid with alcohol, cocaine and/or opioid dependence and relatively small, making the effects of the homozygous minor allele difficult to estimate. The associations we report should be considered in this light when this gene is studied in other populations. Also, we limited our analysis to a single SNP to reduce the penalty for multiple tests. A more comprehensive analysis of SNPs, rare and common, may uncover other risk variants. Characterizing the function of all of these variants might give insight into the relationship between *MANEA* function and anxiety. However, given the LD structure of the gene, larger samples would be required to dissect independent effects on risk. A strength of this study is that it provides evidence for an association between *MANEA* and anxiety risk in multiple independent populations. However, because the third study used very different recruitment criteria and focused on subjects with a different, albeit related, anxiety disorder (social anxiety disorder), we were unable to provide an exact replication of the anxiety disorder phenotype. Moreover, the genetic risk associated with anxiety disorders is not necessarily bound to specific diagnostic criteria, and it is possible that the effects associated with *MANEA* may overlap different disorders.^{34–36} The findings reported here warrant replication aimed at refining the anxiety phenotype that is associated with *MANEA* variation.

In summary, our findings suggest a novel pathway that, when affected, could lead to the development of anxiety disorders. Such novel risk pathways warrant attention and further investigation because they could be the source of new therapeutic targets. Additional studies at the genetic, molecular and behavioral levels are needed to further validate and clarify the relationship between *MANEA* and anxiety-related phenotypes.

CONFLICT OF INTEREST

Although not directly relevant to the work presented here, Henry R Kranzler has been a paid consultant for Alkermes, Lundbeck, Roche, Pfizer and Lilly. He also reports associations with Lilly, Janssen, Schering Plough, Lundbeck, Alkermes, GlaxoSmithKline, Abbott and Johnson & Johnson, as these companies provide support to the American Society of Clinical Psychopharmacology's Alcohol Clinical Trials Initiative (ACTIVE) and Dr Kranzler receives support from ACTIVE. The remaining authors declare no conflict of interest.

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