Association of Variants in MANEA With Cocaine-Related Behaviors

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Context: Cocaine dependence (CD) and related behaviors are highly heritable, but no genetic association has been consistently demonstrated. A recent genome-wide study of drug dependence identified an association between cocaine-induced paranoia (CIP) and a single-nucleotide polymorphism (SNP) in the α-endomannosidase (MANEA) locus in a family-based sample of European Americans and African Americans.

Objective: To conduct a comprehensive genetic association study of the MANEA locus with CD and CIP.

Design: Genome-wide association study.

Setting: Four university hospitals.

Participants: A total of 3992 individuals from 2 family-based and 2 case-control samples.

Intervention: Participants were classified as having CD or CIP or as a control using the Semi-Structured Assessment for Drug Dependence and Alcoholism. They were genotyped for 11 SNPs spanning MANEA and its surrounding region.

Main Outcome Measure: Association of CD and CIP with individual SNPs and haplotypes.

Results: Cocaine-induced paranoia was associated with 6 SNPs in the European American families and 9 SNPs in the African American families. The strongest evidence in the total sample of families was observed in 3 markers located in the promoter and 3’ untranslated regions (P < .001). The association of MANEA SNPs with CD in both family samples was much weaker. In the African American case-control sample, multiple markers were significantly associated with CIP and CD; CIP and CD were also significantly associated with a 2-SNP haplotype in the European American case-control sample. The A allele of the 3’ untranslated region SNP rs9387522 was associated with increased risk of CIP in all 4 data sets.

Conclusions: Our findings suggest that CD and associated behaviors may involve biological pathways not typically thought to be associated with brain metabolism.

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dispose individuals to a number of high-risk behaviors, including accidents, self-harm, and violence toward others.\textsuperscript{19,20}

A genome-wide linkage scan detected regions that harbor genes for CD on chromosomes 3 and 10, and for CIP on chromosome 9,\textsuperscript{21} but no genes under these linkage peaks have yet been identified as risk loci for either condition. Moreover, studies targeting candidate genes selected because of inferred roles in cocaine metabolism or compulsive use have not yet yielded confirmed associations for CD or CIP, though there are several previous reports of CIP’s association with dopamine-pathway genes.\textsuperscript{22,23} Recently, we conducted a genome-wide association study using a low-density SNP array for 6 traits corresponding to 4 major substance dependence disorders (including CD) in a family-based cohort that included 2 distinct population groups. The most remarkable result was an association of a single-nucleotide polymorphism (SNP), rs1133503, in the 3’ untranslated region (UTR) of the MANEA gene (GenBank 79694) with CIP in European American (EA) families (\(P = .007\), African American (AA) families (\(P = .002\), and all families combined (\(P < .001\)).\textsuperscript{24} Although this result was not significant after adjustment for multiple comparisons, the hypotheses that were generated prompted a more comprehensive association study of this gene with cocaine-related traits in 2 discovery data sets (EA and AA families) and 2 independent EA and AA replication data sets composed of unrelated cases and controls ascertained for studies of alcohol and drug dependence.

\section*{METHODS}

\textbf{SUBJECTS}

Subjects were recruited from Yale University School of Medicine (APT Foundation, New Haven, Connecticut), the University of Connecticut Health Center (Farmington), McLean Hospital (Harvard Medical School, Belmont, Massachusetts), and the Medical University of South Carolina (Charleston) into 1 of 2 study arms. Six hundred thirty-two families ascertained through affected sibling pairs that met DSM-IV criteria for CD or opioid dependence as previously described,\textsuperscript{19,20,23} containing at least 1 examined sibling with CD or CIP, formed the discovery sample. Of the 632 families, 119 had at least 1 sibling discordant for CD and 319 had at least 1 sibling pair discordant for CIP. Of the 1612 genotyped subjects, 160 were parents (9.9\%) (and the remainder were siblings) and 141 did not contribute information about substance dependence (8.7\%). An independent group of 2073 unrelated subjects recruited for studies of CD (\(n = 667\)), opioid dependence (\(n = 103\)), or alcohol dependence (\(n = 1303\)) were included in a replication sample. Genetic studies of CD and related traits in a subset of this sample have been published.\textsuperscript{10,23}

All subjects were interviewed using the Semi-Structured Assessment for Drug Dependence and Alcoholism, which has been shown to yield reliable substance dependence diagnoses.\textsuperscript{21,22} Subjects with a primary diagnosis of a major psychotic illness (schizophrenia or schizoaffective disorder) were excluded. A diagnosis of CD was established if the subject met 3 or more of the 7 DSM-IV criteria during a 12-month period. The interrater reliability of the Semi-Structured Assessment for Drug Dependence and Alcoholism diagnosis of CD was previously shown to be \(\kappa = 0.83\).\textsuperscript{21} Subjects who gave an affirmative answer to the question, “Have you ever had a paranoid experience when you were using cocaine?” were diagnosed as being affected by CIP. The overall reliability of CIP diagnosis was previously shown to be excellent (\(\kappa = 0.87\)).\textsuperscript{21} Controls did not use cocaine, but individuals who had dependence on other substances were included. Probands were excluded from further study if they had a diagnosed major psychotic illness (eg, schizophrenia or schizoaffective disorder). Subjects who had missing responses to items on the Semi-Structured Assessment for Drug Dependence and Alcoholism that were required for the determination of CD or CIP status were also excluded.

Subjects gave informed consent as approved by the institutional review board at each clinical site. A certificate of confidentiality for the work was obtained from both the National Institute on Drug Abuse and the National Institute on Alcohol Abuse and Alcoholism. Characteristics of both samples included in this study are given in \textbf{Table 1}.

\section*{SNP SELECTION AND GENOTYPING}

Eleven SNPs spanning 83 kilobases (kb) upstream of the MANEA 3’ UTR were selected from the National Center for Biotechnology Information database or by the Applied Biosystems SNPbrowser, version 3.5 (Applied Biosystems, Foster City, California). Characteristics of each SNP are presented in \textbf{Table 2}. The average intermarker distance is 8293 base pairs (bp) for all SNPs, but only 5600 bp for the 7 SNPs in the promoter and coding regions. Most SNPs were genotyped with a fluorogenic 5’ nucleic as-
samples was evaluated using the FBAT program, assuming no association of the region.

FAMILY-BASED ANALYSES

In the EA family sample, 6 of the 11 markers, including rs1133503 from the genome scan, showed at least a nominally significant association with CIP (Table 3). These 6 markers and 3 others were also significant in the AA family sample. The patterns of association were identical in the 2 population groups for all 9 markers (which represent all markers tested in the promoter and coding regions), evidenced by increased significance in the total sample of families. The strongest evidence for association in either population (AA, \( P < .001 \)) and in the total sample (\( P < .001 \)) was observed with rs6937479, which is located in the putative promoter region. The association of MANEA SNPs with CD was much weaker. In the EA families, nominally significant results were obtained with rs9374586 (\( P = .01 \)), rs1133503 (\( P = .04 \)), and rs9387522 (\( P = .03 \)). Although no significant associations were obtained in the AA families, trends were evident in that population for several markers. Eight markers (rs9400554, rs9320497, rs6937479, rs9374586, rs9400893, rs1133503, rs9387522, and rs9387605) were nominally associated with CD in the total group of families (\( .007 < P \leq .01 \)).

Haplotype analysis was conducted in the family samples to help narrow the location of a CIP susceptibility locus and to determine whether a single functional variant could explain the pattern of association findings with individual SNPs in each population group. As a first step, we evaluated linkage distribution among the 11 SNPs to reduce the number of potentially informative markers for haplotype analysis. This analysis, shown in the Figure, revealed slightly more extensive linkage distribution in EAs than in AAs. These population-
specific patterns are consistent with the linkage distribution structures reported in the HapMap database for this genetic region. Taking into account the linkage distribution block structure and the association findings with individual SNPs, we selected 3 SNPs (rs9400554, rs937479, and rs9387522) for haplotype analysis. These markers include the 2 most significant results in the combined sample and account for the potentially uniquely important information from each linkage distribution block spanning the entire region, showing significance with any marker in either population sample. The haplotype that included this SNP combination was significantly associated in AAs (global, \( P = .003 \)), EAs (global, \( P = .02 \)), and the combined sample of families (global, \( P = .001 \)). The specific haplotype T-T-A was associated with CIP in EAs (\( P = .01 \)) and AAs (\( P = .02 \)), and in the pooled sample (\( P < .001 \)). Haplotype C-A-C was associated with decreased risk of CIP in EAs (\( P = .01 \)) and AAs (\( P < .001 \)), and in the total sample (\( P < .001 \)). These 2 haplotypes account for 86% and 73% of all haplotypes in the EA and AA families, respectively. A third haplotype (C-T-A), which had appreciable frequency in both EAs (6%) and AAs (23%), was also associated with increased risk of CIP. Because both rs9400554 alleles were part of different risk haplotypes, the functional variant is more likely to be closer to the other 2 SNPs. Thus, these results, showing strong evidence for association of the same haplotype to CIP in 2 distinct populations, support the existence of a single causative variant that is most likely located in the MANEA promoter or coding region.

### CASE-CONTROL ANALYSIS

We evaluated the panel of MANEA SNPs in the EA and AA case-control samples in an attempt to replicate the overall association with cocaine-related traits, to determine whether or not the association is specific to CIP, and to localize the putative biological variant. In the AA replication sample, significant association at the allelic and/or genotypic level was observed between CIP and 5 markers (Table 4). These SNPs and a sixth marker were also associated with CD in the absence of CIP. The strongest and most consistent evidence for association was observed with adjacent markers rs9387522 and rs9387605. In the EA replication sample, the only significant association was found for rs4388292 with CIP, which is accounted for primarily by an underrepresentation of the TT genotype in CIP cases compared with controls. The TT genotype is interestingly also significantly lower in

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**Table 3. Association of MANEA SNPs With CIP in Discovery Samples**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Minor Allele</th>
<th>MAF</th>
<th>Families, a No.</th>
<th>P Value</th>
<th>Risk Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9400554</td>
<td>T</td>
<td>0.442</td>
<td>82</td>
<td>.16</td>
<td></td>
</tr>
<tr>
<td>rs10782175</td>
<td>A</td>
<td>0.344</td>
<td>59</td>
<td>.64</td>
<td></td>
</tr>
<tr>
<td>rs9320497</td>
<td>A</td>
<td>0.441</td>
<td>70</td>
<td>.002b</td>
<td>T</td>
</tr>
<tr>
<td>rs937479</td>
<td>A</td>
<td>0.483</td>
<td>77</td>
<td>.002b</td>
<td>T</td>
</tr>
<tr>
<td>rs9374586</td>
<td>T</td>
<td>0.385</td>
<td>76</td>
<td>.07</td>
<td>C</td>
</tr>
<tr>
<td>rs9400893</td>
<td>A</td>
<td>0.440</td>
<td>74</td>
<td>.01b</td>
<td>G</td>
</tr>
<tr>
<td>rs7757276</td>
<td>G</td>
<td>0.130</td>
<td>31</td>
<td>.46</td>
<td></td>
</tr>
<tr>
<td>rs1135363</td>
<td>C</td>
<td>0.439</td>
<td>80</td>
<td>.007b</td>
<td>T</td>
</tr>
<tr>
<td>rs937622</td>
<td>C</td>
<td>0.437</td>
<td>71</td>
<td>.003b</td>
<td>A</td>
</tr>
<tr>
<td>rs9387605</td>
<td>A</td>
<td>0.445</td>
<td>70</td>
<td>.03b</td>
<td>G</td>
</tr>
<tr>
<td>rs4388292</td>
<td>T</td>
<td>0.200</td>
<td>45</td>
<td>.45</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** CIP, cocaine-induced paranoia; MAF, minor allele frequency; SNP, single-nucleotide polymorphism.

a Informative families.

b Significant result.

c T allele.
individuals with CD compared with controls in the AA replication sample. Although the results of analyses of individual SNPs did not show an association common to both population groups, haplotype analysis of rs900554 and rs9387522 (ie, 2 of the 3 SNPs included in the haplotype studies in the families) showed that the C-A haplotype was significantly associated with increased risk of CIP and CD in EAs and that the T-A haplotype was significantly associated with CD in AAs (Table 5). The rare T-C haplotype was also associated with increased risk of CD in EAs. Of note, when considering results from both the single SNP and haplotype analyses in the replication samples, the rs9387522 A allele is associated with CIP in all 4 data sets.

**COMMENT**

We observed that several polymorphic markers in the MANEA gene region are associated with cocaine-related traits in 2 EA and 2 AA populations, which were ascertained and analyzed in different ways. The strongest evi-

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**Table 5. Haplotype Association of MANEA With Cocaine-Induced Paranoia in the Replication Samples**

<table>
<thead>
<tr>
<th>Allele by SNP</th>
<th>European Americans</th>
<th>African Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haplotype Frequency</td>
<td>Z Score</td>
</tr>
<tr>
<td>rs900554</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.474</td>
<td>-0.64</td>
</tr>
<tr>
<td>C</td>
<td>0.4</td>
<td>-1</td>
</tr>
<tr>
<td>A</td>
<td>0.12</td>
<td>2.25</td>
</tr>
<tr>
<td>T</td>
<td>0.06</td>
<td>0</td>
</tr>
</tbody>
</table>

**Cocaine Dependence**

<table>
<thead>
<tr>
<th>Allele by SNP</th>
<th>European Americans</th>
<th>African Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Haplotype Frequency</td>
<td>Z Score</td>
</tr>
<tr>
<td>rs900554</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.47</td>
<td>-1.08</td>
</tr>
<tr>
<td>C</td>
<td>0.4</td>
<td>-0.81</td>
</tr>
<tr>
<td>A</td>
<td>0.12</td>
<td>2.25</td>
</tr>
<tr>
<td>T</td>
<td>0.06</td>
<td>2.86</td>
</tr>
</tbody>
</table>

Abbreviations: CD, cocaine dependence; CI, confidence interval; CIP, cocaine-induced paranoia; MAF, minor allele frequency; ND, test not done because controls were not in Hardy-Weinberg equilibrium; OR, odds ratio; SNP, single-nucleotide polymorphism.

a In European Americans: global, P=.04; in African Americans: global, P=.14.

b Significant result.

c In European Americans: global, P=.003; in African Americans: global, P=.06.
dence was obtained for CIP with markers in the MANEA coding and promoter regions, spanning a distance of approximately 33.6 kb (ie, between rs9320497 and rs9387522). Haplotype analysis in the replication data sets helped confirm that the rs9387522 A allele is associated with increased risk of CIP in all 4 populations. This SNP is only 348 bp from rs1133505, the marker in the low-density genome scan that showed an association with CIP in the EA and AA family-based samples,34 which prompted this investigation. Our comprehensive analysis of MANEA SNPs and haplotypes in 4 independent data sets bolsters our initial association finding and indicates that the biologically relevant variant is most likely located in the 3′ UTR.

The results for association of MANEA with CD were substantially weaker in the discovery (family-based) data sets. However, these samples were ascertained through sibling pairs with CD or opioid dependence. They are, thus, much less informative for association analyses of these traits compared with those with CIP, because, in the absence of data from parents, the family-based approach requires at least 1 discordant sibling pair. To determine whether the association with MANEA is specific to the paranoia that often complicates CD, we compared MANEA SNPs and haplotypes in controls with distinct samples of subjects with CD (but no paranoia) and subjects with CIP. In the AA group, CD and CIP were significantly and comparably associated with several SNPs. Both traits showed identical patterns of association with a particular haplotype in the EA group. Thus, our study suggests that MANEA is associated with both CD and paranoia. It is also possible that MANEA is more strongly associated with CIP than CD because CIP is characteristic of a genetic subgroup of CD that is influenced by MANEA. Additional studies in independent samples of subjects with CD characterized for paranoia, and perhaps in subjects with other disorders involving paranoia, are necessary to determine more definitively whether the association with MANEA is specific for the subset of persons with CD prone to CIP.

α-Endomannosidase (MANEA), encoded by the MANEA gene on chromosome 6q16.1, is an enzyme that catalyzes the release of glucosyl-mannose oligosaccharides by cleaving the α-1,2-mannosidic bond that links them to high-mannose N-glycans.35 Comparative genomic analysis has demonstrated high-sequence conservation in humans, rats, and mice.36 Human MANEA is expressed in a variety of tissues including brain, though levels of MANEA in the brain are much lower than, for example, in the liver or kidney.36 The role of MANEA is poorly understood but has been hypothesized to be involved in the quality control of N-glycosylation,37 providing cells with the ability to recover and properly mature glucosylated structures that have bypassed glucosidase trimming by glucosidases I and II in the endoplasmic reticulum.36

Given MANEA’s role in carbohydrate metabolism and its relatively minor expression in brain, initially it would not appear to be a good biological candidate to modulate susceptibility to CD or its associated psychotic complications. However, insight into the relationship between MANEA, paranoia, and CD can be gleaned from studies of mannosidase and other glycoproteins. α-Mannosidosis in humans is a rare autosomal recessive lysosomal storage disorder associated with decreased activity of mannosidase. Recently, α-mannosidosis was identified as the underlying cause of recurrent paranoid hallucinatory episodes in a 27-year-old woman.38 α-Endomannosidase is 1 of several glycosidic enzymes that remove oligosaccharide chains of dopamine β-hydroxylase,39 the enzyme that converts dopamine to noradrenaline. Low levels of β-hydroxylase in plasma or cerebrospinal fluid and polymorphisms in β-hydroxylase have been associated with greater vulnerability to psychotic symptoms in several psychiatric disorders including CD.40,41 schizophrenia,41 and major depression.42 α-Endomannosidase may also influence susceptibility to CD by modifying the function of liver carboxylesterase, a glycoprotein of the high mannose type,43 2 forms of which hydrolyze cocaine and other drugs.44,45

There are 44 markers in MANEA with appreciable frequency in 1 or more populations (http://www.ncbi.nlm.nih.gov/SNP), but none are known to effect structural changes in the translated protein. Remarkably, 34 of these SNPs have minor allele frequencies of 0.24 or greater in both EAs and AAs. This excess of high-frequency polymorphisms suggests that balancing selection is occurring in this region.46 The most robust evidence for association in the collective data sets in this study was obtained with rs9387522, which is located in the 3′ UTR. The 3′ UTR is the major site of gene regulation by microRNA binding.47 Polymorphic target sites for microRNA binding in the 3′ UTR of SLITRK1, FGF20, and HTR1B have been identified as leading to increased risk of Tourette syndrome,48 Parkinson disease,49 and aggressive human behaviors,50 respectively. The possibility that MANEA 3′ UTR SNPs, including rs987522, may influence risk of CD or CIP could be investigated by microRNA studies in brains of rodents exposed to cocaine or constructs transfected into cell lines to demonstrate effects on gene expression.

We acknowledge several limitations to our study. First, our discovery sample, which was ascertained through sibling pairs concordant for CD or opioid dependence, is probably enriched for genetic factors for CD and CIP compared with subjects exposed to cocaine in the general population. To overcome this issue and the problem that association findings in discovery samples tend to overestimate the effect size of the genetic risk factor,51 we replicated our results in independent EA and AA case-control samples. Although results with individual SNPs were uneven across study samples, haplotype analysis showed significant association with the same allele of 1 SNP (SNP 9, rs9387522) in both EA and both AA data sets. Second, genetic association studies are vulnerable to false-positive results owing to population stratification and to false-negative results owing to misclassification of subjects or power. Our use of family-based controls in the discovery phase and the assignment of nearly all subjects to genetically matched groups based on analysis of many markers distributed across the genome lessened the potential for stratification. Furthermore, all of the approximately 4000 subjects included in this study were evaluated with a standardized instrument using a
rigorous quality-control procedure that reliably diagnoses substance dependence and other psychiatric disorders. In any event, it is possible that some subjects were misclassified as controls because they were not sufficiently exposed to cocaine to become dependent on the drug. This, however, would bias the results toward the null hypothesis. Because our replication samples had sufficient power to detect allele frequency differences of 7% to 10% for CIP and 8% to 15% for CD in either population, lack of significant findings with individual SNPs in the EA sample could be attributed to an inadequate sample size. However, significant haplotypic associations in this population suggest that genetic background rather than sample size was the limiting factor. Third, only one of the results from analyses of individual SNPs in the replication samples would be considered significant after adjustment for multiple comparisons using a conservative Bonferroni correction (threshold, $P = 0.04$ in EAs and $P = 0.006$ in AAs without taking intermarker correlations into account). An alternative approach to evaluating the impact of multiple testing on our results is measuring the rate of false discovery. Because the expected number of findings for a trait that surpass a nominal significance level of $P = 0.05$ in the AA sample would be less than 1 (0.05 × 9 informative SNPs × 0.5), assuming a 1-tailed test (and there were at least 3 significantly associated SNPs for each trait, taking into account the high correlation among SNPs 5 through 9 in AAs [Figure 1]), it is unlikely that our findings for CD and CIP in the AA replication sample are spurious. The significant global tests of association of MANEA haplotypes with CD and CIP in the EA replication sample take into account the comparisons of multiple haplotypes. In summary, our study shows that MANEA gene variants are strongly associated with CD and CIP in both EA and AA populations. This finding, which was discovered initially through a low-density genome scan, suggests that drug dependence and associated behaviors may involve biological pathways not typically associated with brain metabolism and opens a new pathway to understanding these highly prevalent disorders and their psychopathologic manifestations.

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