

Interaction between Two Independent *CNR1* Variants Increases Risk for Cocaine Dependence in European Americans: A Replication Study in Family-Based Sample and Population-Based Sample

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We recently reported that, in a European-American (EA) sample, the interaction between two cannabinoid receptor 1 gene (*CNR1*) variants significantly increased risk for drug dependence (DD), including cocaine dependence (CD). This study aimed to investigate directly the association between *CNR1* and CD in four independent samples. Eight markers across the 45 kb *CNR1* region and four large samples, ie, family-based European-American (EA) sample ($n = 734$), case–control EA sample ($n = 862$), family-based African-American (AA) sample ($n = 834$) and case–control AA sample ($n = 619$) were examined in the present study. We investigated the association of these markers with CD and cocaine-induced paranoia (CIP) in the EA family sample first, and then replicated positive results in the other three samples. The interaction between two independent *CNR1* variants, ie, the G allele-containing genotypes of rs6454674 (SNP3^{G+}), and the T/T genotype of rs806368 (SNP8^{T/T}), significantly increased risk for CD in the EA family ($P_{GEE} = 0.015$) and EA case–control ($P_{\text{regression}} = 0.003$) samples. EA subjects with SNP3^{G+} and SNP8^{T/T} had higher risk to develop CD than those EA subjects with the other genotypes for these two SNPs ($LR^+ = 1.4$). The SNP3^{G+}-SNP8^{T/T} haplotype also showed significant association ($P = 0.018$) with CD in the EA case–control sample. SNP8-containing haplotypes showed significant association with both CD ($P_{\text{global}} = 0.007$) and CIP ($P_{\text{global}} = 0.003$) in the EA family sample. In the AA family sample, SNP8^{T/T} significantly conferred higher risk for CD ($P = 0.019$). We conclude that two independent *CNR1* variants have significant interaction effects on risk for CD in EAs; they may also have effects on risk for CD in AAs.

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INTRODUCTION

Cocaine dependence (CD), which occurs commonly in the United States, is characterized by compulsive cocaine seeking and continued cocaine use despite health problems.

Cocaine-dependent individuals are at high risk of relapse to heavy cocaine use even after a period of abstinence. Limited efficacy of prevention and treatment efforts for CD are attributable in part to the unclear etiology of CD. Genetic epidemiological studies support a genetic contribution to the risk for CD (discussed in (Gelernter *et al*, 2005)). However, to date, few genetic variants have been confirmed as contributing to this vulnerability.

The cannabinoid receptor 1 gene (*CNR1*) has emerged as a promising contributor to CD vulnerability. *CNR1* is located in 6q14–15 and encodes a seven transmembrane signaling protein, ie, the cannabinoid receptor 1 (CB1),

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which is preferentially distributed in the presynaptic membrane of neurons (Di Marzo *et al*, 2004). δ -9-Tetrahydrocannabinol (THC), the main active ingredient in marijuana, is an exogenous ligand for CB1. Anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) are the two major known endogenous ligands for CB1. Both AEA and 2-AG act as retrograde messengers, moving backward across the synapse from the postsynaptic neuron, and binding to CB1 to depress neurotransmitter release either transiently or over a longer time course (Chevalyere *et al*, 2006; Lupica and Riegel 2005). Neurobiological studies show that CB1 is one of the most abundant neuromodulators in the mammalian brain, including neocortex, hippocampus, basal ganglia, cerebellum, striatum, and the ventral tegmental area (VTA) (Arnold 2005; Di Marzo *et al*, 2004; Herkenham *et al*, 1990; Mailleux and Vanderhaeghen 1992; Solinas *et al*, 2008; Tsou *et al*, 1998).

Supporting possible relevance to CD, many lines of evidence show that CB1 and its ligands are involved in the regulation of mesocorticolimbic dopamine (DA) reward pathways that project from VTA to nucleus accumbens (NAc), prefrontal cortex (PFC), amygdala, and hippocampus. The mesocorticolimbic DA reward pathways are the main hypothesized pathways for addiction, including CD (Di Chiara and Imperato, 1988; Koob, 1992; Koob and Le Moal, 2001; Koob and Le Moal, 2008; Lupica and Riegel, 2005; Nestler, 2005; Tanda and Goldberg, 2003). Although all classes of commonly abused substances activate the mesocorticolimbic system, the mechanisms of this activation differ across abusable substances. In contrast to opioids, nicotine, or alcohol, the main receptor for cocaine is the DA transporter, to which cocaine binds, resulting in the suppression of DA reuptake, with a subsequent increase in extracellular DA level (Hyman *et al*, 2006). Some studies suggested that CB1 might not be important in cocaine self-administration (Caille *et al*, 2007; Cossu *et al*, 2001), but the function of the DA system on the effects of cocaine and the modulatory function of the endocannabinoid system in DA reward pathways suggest that CB1 may be involved in the risk of developing CD.

Consistent with evidence of a potential shared mechanism of risk, marijuana dependence (MD) frequently co-occurs with CD (Miller *et al*, 1990). Further, cocaine administration increases levels of AEA in striatum. This effect decreases by pharmacological inhibition of DA-2-like receptors (Centonze *et al*, 2004). Besides the reduction effect on AEA release by pharmacological inhibition of D2-like receptors, administration of D2-like receptor agonists increased AEA release (Giuffrida *et al*, 1999). In addition, blockade of CB1 receptors can partially prevent the inhibitory effect of cocaine on GABA transmission (Centonze *et al*, 2004). Blockade of CB1 can also decrease the DA signal induced by cocaine in NAc (Cheer *et al*, 2007). Endocannabinoid system-mediated synaptic plasticity, eg, long-term potentiation (eCB-LTP) and long-term depression (eCB-LTD), have been identified in brain (Carlson *et al*, 2002; Chevalyere *et al*, 2006), and the endocannabinoid system is involved in the mediation of cocaine-induced LTD in midbrain DA neurons (Pan *et al*, 2008). Animal studies further have shown that, after a prolonged withdrawal period, pretreatment with a CB1 antagonist (SR141716) attenuates cocaine relapse induced by exposure

to cocaine or cocaine-related cues, whereas pretreatment with a CB1 agonist (HU210) precipitates a relapse to cocaine-seeking behavior (De Vries *et al*, 2001). Finally, the motivation to seek cocaine is decreased in CB1 knockout mice compared to wild type mice (Soria *et al*, 2005).

Recently, in a European-American (EA) sample, we observed that the interaction between two variants of *CNR1* significantly increased risk for drug dependence (DD), which included CD and/or OD (Zuo *et al*, 2007). This study further investigated the association between *CNR1* and CD. Moreover, a related phenotype, ie, cocaine-induced paranoia (CIP), was included to increase the information gained from the present study (Gelernter *et al*, 2005). Genetic variants in the dopamine transporter gene (*DAT1*) and the dopamine β -hydroxylase gene (*DBH*) were reported to confer genetic vulnerability to CIP (Cubells *et al*, 2000; Gelernter *et al*, 1994; Kalayasiri *et al*, 2007). We have recently reported several additional associations based on a low-density genome-wide association scan (Yu *et al*, 2008).

MATERIALS AND METHODS

Subjects

Four independent samples, ie, the EA family sample, the EA case-control sample, the African-American (AA) family sample, and the AA case-control sample, were included in this study. All subjects gave written informed consent as approved by the relevant Institutional Review Boards. The EA family sample ($n = 734$) included 307 pedigrees (551 CD cases and 394 CIP cases). The AA family sample ($n = 834$) included 316 pedigrees (664 CD cases, 432 CIP cases). The EA case-control sample ($n = 862$) included 299 CD cases and 563 healthy controls. The AA case-control sample ($n = 619$) included 406 CD cases and 213 healthy controls. Of the case-control samples, 175 EA CD cases, 403 EA controls, 136 AA CD cases and 48 AA controls were included in our initial study (Zuo *et al*, 2007). Demographic characteristics are listed in Table 1.

All CD cases met lifetime DSM-III-R or DSM-IV criteria for CD (APA, 1987, 1994). Individuals with schizophrenia or another psychotic illness were excluded. CIP was defined as having a transient, unrealistic, and self-limited paranoid experience, eg, irrational intense suspicion of others. CIP occurs during cocaine use and typically resolves with abstinence. CIP was assessed only among individuals with a diagnosis of CD. The controls were screened to exclude major axis I disorders, including substance dependence (SD), schizophrenia, mood disorder, major anxiety disorder and more, using DSM-III-R, DSM-IV, and the Schedule for Affective Disorders and Schizophrenia (SADS) (Spitzer and Endicott, 1975). The subjects were recruited from four sites: Yale University School of Medicine, University of Connecticut Health Center, McLean Hospital of Harvard Medical School, and Medical University of South Carolina.

Marker Selection, Genotyping, and Error Checking

Eight markers across the 45 kb region in *CNR1* were selected (Figure 1). From 5' to 3' of *CNR1*, the 8 markers were numbered as SNPs 3–10 (matching their designation in our initial study (Zuo *et al*, 2007)). They were selected from

Table 1 Demographic Characteristics of Four Samples

Sample type	Characteristics	EAs	AAs
Family sample	No. of pedigrees	307	316
	No. of pedigrees with 1 CD	81	40
	No. of pedigrees with 2 CD	200	211
	No. of pedigrees with 3 CD	24	55
	No. of pedigrees with 4 CD	2	7
	No. of nuclear families	277	242
	No. of total subjects	734	834
	Male (%)	51.0	41.0
	Age (mean \pm SD)	37.9 \pm 10.3	40.8 \pm 7.3
	No. of total CD	551	664
No. of CIP	394	432	
Population sample	No. of total CD (No. in initial study ^a)	299 (175)	406 (136)
	No. of control (No. in initial study)	563 (403)	213 (48)
	% male CD	68.6	63.1
	Age of CD (mean \pm SD)	38.1 \pm 8.7	40.9 \pm 8.1
	% male controls	44.6	28.2
	Age of controls (mean \pm SD)	30.4 \pm 11.2	35.2 \pm 13.2

AA, African American; CD, cocaine dependence; CIP, cocaine-induced paranoia; EA, European American.

^aSubjects involved in our initial study.

the NCBI dbSNP database or from the Applied Biosystems SNP database. Marker selection was based on published data, minor allele frequency, information content, LD structure, region coverage, and potential function.

Genomic DNA was obtained from immortalized cell lines, blood or saliva. All SNPs were genotyped by a fluorogenic 5' nuclease assay (TaqMan Technique (Shi *et al*, 1999)). Mendelian inconsistencies in family genotype data were identified by implementing Pedcheck (O'Connell and Weeks, 1998). One inconsistency was found and the genotype data for this entire pedigree were excluded from this study. Hardy-Weinberg equilibrium (HWE) was tested using the program PowerMarker (Liu and Muse, 2005).

Study Design

Genetic populations were identified through Bayesian clustering using STRUCTURE (Gelernter *et al*, 2005; Yang *et al*, 2005). Because family-based association tests (FBATs) avoid confounding effects from model misspecification and population stratification or admixture (Laird *et al*, 2000), we performed association tests in the EA family sample first, and then replicated positive results in the other three independent samples. To exclude the potential confounding effects on CD from other subtypes of comorbid SD, we adopted a one-by-one exclusion strategy described below.

Family-Based Association Test

The FBAT program was used to perform association tests in the family sample (Laird *et al*, 2000). FBAT was developed on a principle similar to the transmission disequilibrium

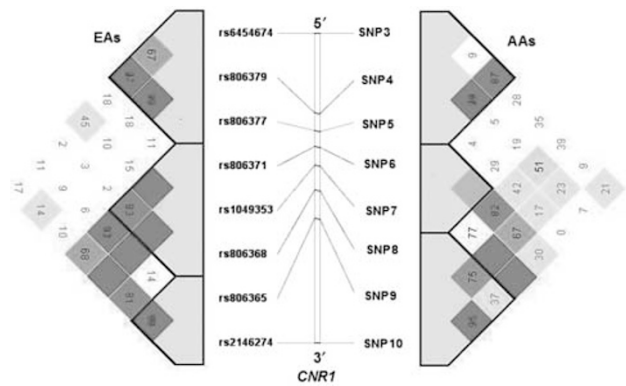


Figure 1 Pairwise linkage disequilibrium (LD) of cannabinoid receptor 1 (*CNR1*) markers in family samples. LD blocks were detected by haploview v3.32 and haplotype blocks were defined by solid spine of LD (Barrett *et al*, 2005). EAs and AAs represent European Americans and African Americans, respectively; the numbers inside the square are $D' \times 100$; the blank dark squares indicate $D' = 1$.

test (TDT), which controls for population stratification by comparing the alleles transmitted by parents to affected offspring with the nontransmitted alleles (Spielman *et al*, 1993). The null hypothesis is 'no linkage and no association' or 'no association, in the presence of linkage'. The FBAT is a more general test that avoids confounding due to model misspecification as well as admixture or population stratification (<http://www.biostat.harvard.edu/~fbat/default.html>).

Genetic EA and AA ancestries for the family sample were assigned based on our earlier study (Gelernter *et al*, 2005) and all of the analyses in this study were performed in genetic EAs and AAs separately.

Haplotype associations were tested by the program HBAT in the FBAT package (Laird *et al*, 2000). We adopted the global haplotype association test with Monte Carlo simulation, ie, repeated random sampling, to compute an exact global *P*-value for multihaplotype-based association (<http://www.biostat.harvard.edu/~fbat/default.html>).

Case-Control Association Test

Allele-wise and genotype-wise associations were tested using Fisher's exact test by comparing allele and genotype frequency distributions between cases and controls. Haplotype trend regression (HTR) analysis was performed using Powermarker through permutation (permutation number was set as 100 000) by comparing the haplotype frequency distributions between cases and controls. The haplotype simulation association test was implemented using the program Haplo.score in the Haplo.stats package (<http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm>), with sex and age as covariates.

The case-control design is vulnerable to population stratification and admixture effects. To control for these effects, we also performed structured association analysis. First, we estimated the ancestry proportions for each unrelated subject, based on the information content of 37 AIMs using the program STRUCTURE (Pritchard *et al*, 2000a). Parameters for burn-ins, iterations, and *k* were set

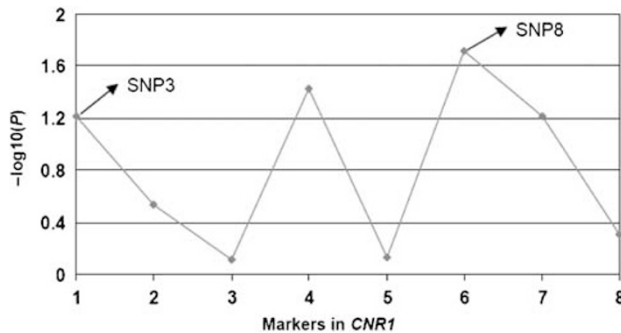


Figure 2 $-\log_{10}(P)$ values for the associations of cocaine dependence (CD) with 8 markers in cannabinoid receptor 1 (*CNR1*) in European-American (EA) family sample. Markers 1–8 represent rs6454674 (SNP3), rs806379 (SNP4), rs806377 (SNP5), rs806371 (SNP6), rs1049353 (SNP7), rs806368 (SNP8), rs806365 (SNP9), and rs2146274 (SNP10), respectively.

as 100 000, 100 000, and 2, respectively. STRAT (Pritchard *et al*, 2000b) was used to analyze allele-wise and genotype-wise associations between the candidate markers and CD by excluding the admixture effects. We performed 10 000 simulation iterations per marker.

Marker–Marker Interaction

Genotypes within pedigrees are correlated. The generalized estimating equations (GEEs) method (Liang and Zeger, 1986; Zeger and Liang, 1986), an extension of the quasi-likelihood approach (Wedderburn, 1974), is used to analyze correlated data, longitudinal data, and family data (Chen *et al*, 2004; Hanley *et al*, 2003; Lange *et al*, 2003). In this study, GEE as implemented in SPSS v15 (SPSS Inc.), was implemented to model CD using a two-way interaction between the risk SNP and other SNPs of *CNR1*. The model applied a logit link function and Pearson's χ^2 estimating method, while adjusting for sex and age. The working correlation matrix was set as unstructured, and maximum iteration was set as 100 000. For GEE there is no prior assumption for data distribution, and as the number of unaffected in our family sample was limited, for GEE analysis, we increased the control sample size by pooling 403 EA controls and 48 AA controls from our case–control samples with the corresponding family samples.

Marker–marker interaction analyses in case–control sample were performed by mixed model backward logistic regression (Zuo *et al*, 2007). The phenotypes served as the dependent variable and sex, age, and genotypes were covariates.

Linkage Disequilibrium

The dependence of allele frequencies at two loci is called linkage disequilibrium (LD) (Lonjou *et al*, 2003). D' (the value of LD normalized to the highest observable value between the two loci considered) values for pairwise LD of *CNR1* markers were calculated and visualized through the program haploview v3.32 (Barrett *et al*, 2005).

RESULTS

Associations of *CNR1* with CD in the EA Family Sample

Associations between CD and the eight SNPs in *CNR1* in the EA family sample are shown in Figure 2. rs806368 (SNP8) showed the most significant association signal ($P = 0.019$). The second most significant association signal was for rs806371 (SNP6, $P = 0.038$), which showed almost as high a δ peak as SNP8 in the fine-mapping analysis in our initial study (δ is a measure of LD used in fine-mapping analysis) (Zuo *et al*, 2007). SNP6 is located within the SNP8-containing haplotype block and is correlated with SNP8 ($D' = 0.84$, $r^2 = 0.49$). Thus, the CD association signal at SNP6 is consistent with the association between SNP8 and CD, and also suggests that SNP6 may help to capture the association signal of SNP8 locus. The third most significant CD association signal was seen for rs6454674 (SNP3), though this did not reach statistical significance ($P = 0.060$). No other association signal was observed.

In the global haplotype association test, the frequency distribution of SNP8-containing haplotypes (constructed from three SNPs within the haplotype block that contains SNP8) in CD cases differed significantly from controls ($P_{\text{global}} = 0.007$, with $\alpha = 0.017$). Moving the haplotype window in the 5' or 3' direction in *CNR1*, this association signal decreased (data not shown). We failed to observe an association between SNP3-containing haplotypes and CD.

On the basis of the findings in our initial study and on the significant association signal observed here at SNP8, we performed an interaction analysis between SNP8^{A/T/T} and SNP3^{G/G} (G/G and G/T for SNP3), which conferred significantly higher risk for CD ($P = 0.015$; Table 2). In addition, exhaustively analyzing two-way marker–marker interactions showed that SNP8^{A/T/T} had no significant interaction with the other markers (except for SNP3) in *CNR1* ($P > 0.05$). As shown in Figure 1, SNP3 and SNP8 are neither in LD ($D' = 0.026$) nor correlated with each other ($r^2 = 0$).

CD is highly comorbid with other SD (Kranzler *et al*, 2008). In our family sample, the majority of CD cases were comorbid with another SD, including 77.0% with nicotine dependence (ND), 64.2% with opioid dependence (OD), 52.1% with alcohol dependence (AD), 35.9% with MD and 26.0% with other types of SD (R-SD). Because of the high degree of comorbidity, it was not feasible to study a 'CD-only' phenotype (4%, $n = 22$) (nor is this phenotype important clinically, compared to comorbid phenotypes). Consequently, we excluded different types of comorbid SD one-by-one to control for the potential confounding effects from other SD (Figure 3). This one-by-one exclusion created five subgroups of CD, ie, CDnoAD (CD without comorbid AD), CDnoOD (CD without comorbid OD), CDnoMD (CD without comorbid MD), CDnoND (CD without comorbid ND), and CDnoR-SD (CD without comorbid R-SD). After excluding the effects of AD, OD, MD, and R-SD, the association signal remained significant for SNP8 ($P_{\text{CDnoAD}} = 0.012$; $P_{\text{CDnoOD}} = 0.021$; $P_{\text{CDnoMD}} = 0.014$; $P_{\text{CDnoR-SD}} = 0.020$). After excluding ND, the association signal for CDnoND decreased; a 'trend-level possible' association signal remained ($P = 0.071$). At SNP3, the 'trend-level possible' association signals ($P = 0.060$)

Table 2 Interaction Effects of SNP3^{G⁺} and SNP8^{T/T} on CD in EA Family Sample and EA Case–Control Sample

Parameter	EA family sample		EA case–control sample	
	β	P-value	β	P-value
Sex	0.62	3.14×10^{-5}	0.93	1.3×10^{-7}
Age	0.10	5.29×10^{-16}	0.07	4.2×10^{-16}
SNP3 ^{G⁺} × SNP8 ^{T/T} ^b	0.29	0.015	0.42	0.003

^aG/G and G/T genotype for SNP3.

^bSNP3^{G⁺} × SNP8^{T/T} denotes the interaction between G/G and G/T genotypes of SNP3 and T/T genotype of SNP8.

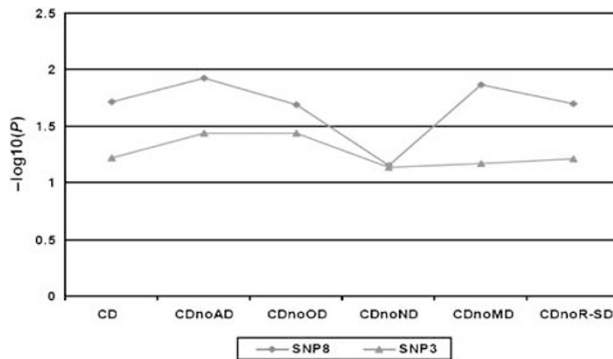


Figure 3 $-\log_{10}(P)$ values of associations of SNP3 and SNP8 with six different cocaine dependence (CD) subgroups in the European-American (EA) family sample. CDnoAD, CDnoOD, CDnoMD, CDnoND, and CDnoR-SD represent CD without comorbid alcohol dependence, CD without comorbid opioid dependence, CD without comorbid marijuana dependence, CD without comorbid nicotine dependence, and CD without comorbid rest type of substance dependence, respectively.

remained or became significant after excluding the effect of AD ($P_{\text{CDnoAD}} = 0.036$), OD ($P_{\text{CDnoOD}} = 0.036$), ND ($P_{\text{CDnoND}} = 0.072$), MD ($P_{\text{CDnoMD}} = 0.068$), or R-SD ($P_{\text{CDnoR-SD}} = 0.062$).

Associations of CNRI with CIP in EA Family Sample

For CIP, nominally significant association signals were observed at two SNP8-linked loci, ie, rs1049353 (SNP7) ($P = 0.015$) and rs2146274 (SNP10) ($P = 0.010$). SNP3 and SNP8 were not significantly associated with CIP ($P_{\text{SNP3}} = 0.081$, and $P_{\text{SNP8}} = 0.071$). In the global haplotype association test, the SNP8-containing haplotype showed a highly significant association with CIP ($P = 0.003$), which remained significant after correcting for multiple testing (with $\alpha = 0.017$). These findings suggest that the SNP8 locus may also be important in CIP. However, we failed to observe an interaction effect between SNP3^{G⁺} and SNP8^{T/T} on CIP.

Associations of CD with the Positive Variants Identified in the EA Family Sample in the Other Three Independent Samples

The above EA FBATs suggested that SNP8 and the interaction between SNP8 and SNP3 were associated with risk for CD. We replicated these positive associations using

the other three independent samples, ie, an EA case–control sample, an AA case–control sample and an AA family sample.

In the EA case–control sample, SNP3 and SNP8 were in HWE in both cases and controls. In the AA case–control sample, SNP3 was in significant Hardy–Weinberg disequilibrium (HWD) in cases ($P = 7.0 \times 10^{-4}$), and SNP8 was in nominal HWD in cases ($P = 0.025$), but both were in HWE in controls ($P > 0.05$), which may be an indication of marker–phenotype association (Luo *et al*, 2006).

The allele and genotype frequencies of SNP3 and SNP8 in EA and AA case–control samples are listed in Table 3. In controls, we observed that the G allele frequency of SNP3 and T allele frequency of SNP8 in AAs were significantly higher than those in EAs ($P_{\text{SNP3}} = 0.006$, $P_{\text{SNP8}} = 4.5 \times 10^{-10}$).

In the single-marker-wise analysis in EAs, we failed to observe that allele and genotype frequency distribution for SNP3 or SNP8 was significantly different from controls (Table 3). However, as expected, in the EA case–control sample, the interaction between SNP3^{G⁺} and SNP8^{T/T} significantly increased risk for CD ($P = 0.003$) while adjusting for sex and age. This is consistent with our initial study, which showed that this interaction significantly increased risk for DD ($P = 0.0002$), including CD (Table 2). Through permutation, HTR analysis using the Powermarker program showed a trend-level possible association between CD and the global haplotypes composed of these two SNPs ($P_{\text{global}} = 0.069$). Haplotype simulation association tests using Haplo.stats showed that global haplotypes and the GT haplotype (constructed from SNP3^G and SNP8^T) displayed significant association signals ($P_{\text{global}} = 0.054$, $P_{\text{GT}} = 0.018$), adjusting for sex and age.

In subjects carrying the SNP3^G allele, the frequency of SNP8^{T/T} in CD cases was significantly higher than controls ($P = 0.008$, OR = 1.8, 95% CI: 1.1–2.9), whereas no significant difference was observed within subjects not carrying SNP3^G. On the other hand, in CD subjects carrying SNP8^{T/T}, the frequency of SNP3^{G⁺} was nominally higher than in controls ($P = 0.024$, OR = 1.6, 95% CI: 1.0–2.4), but no difference was observed within subjects not carrying SNP8^{T/T} ($P > 0.05$) (Table 4; Figure 4).

In the AA family sample, we observed that SNP8^{T/T} significantly increased risk for CD ($P = 0.019$) while adjusting for sex and age. However, neither SNP3 nor the interaction between SNP3^{G⁺} and SNP8^{T/T} was significantly associated with CD ($P > 0.05$). In the haplotype-wise

Table 3 Allele and Genotype Frequencies of SNP3 and SNP8 in Case–Control Samples

SNP	rs #	Ethnicity	Allele or genotype	CD cases		Controls		P-value
				No.	Frequency	No.	Frequency	
SNP3	rs6454674	EA	G	183	0.33	289	0.29	—
			T	365	0.67	703	0.71	—
			G/G	33	0.12	46	0.09	—
			G/T	117	0.43	197	0.40	—
			T/T	124	0.45	253	0.51	—
		AA	G	223	0.33	131	0.37	—
			T	445	0.67	221	0.63	—
			G/G	51	0.15	20	0.11	0.003
			G/T	121	0.36	91	0.52	—
			T/T	162	0.49	65	0.37	—
SNP8	rs806368	EA	T	439	0.80	822	0.77	—
			C	109	0.20	244	0.23	—
			T/T	178	0.65	318	0.60	—
			T/C	83	0.30	186	0.35	—
			C/C	13	0.05	29	0.05	—
		AA	T	707	0.90	379	0.91	—
			C	79	0.10	39	0.09	—
			T/T	322	0.82	174	0.83	—
			T/C	63	0.16	31	0.15	—
			C/C	8	0.02	4	0.02	—

‘—’ represent $P > 0.05$.

Table 4 Associations of CD With SNPs 3 and 8 in EA Family Sample and EA Case–Control Sample

SNPs	Family sample		Case–control sample				
	No. of informative families	P	Genotype	CDs	Controls	P	OR
SNP3	31	0.06	G/G+G/T	98	134	0.024 ^a	1.6
			T/T	68	146		
SNP8	33	0.019	T/T	98	134	0.008 ^b	1.8
			T/C+C/C	41	102		

^aP-value for SNP3^{^(G/G+G/T)} vs T/T in subjects carrying the SNP8^{^T/T} genotype.

^bP-value for SNP8^{^T/T} vs (T/C+C/C) in the subjects carrying the SNP3^{^(G/G or G/T)}.

analysis, no association between CD and SNP8-containing haplotypes was observed.

In the AA case–control sample, as shown in Table 3, the allele frequency distributions for both SNP3 and SNP8, and the genotype frequency distribution for SNP8 in cases, were not significantly different from controls. The genotype frequency distribution for SNP3 in cases was significantly different from controls, however the difference did not reach statistical significance while adjusted for sex and age, and corrected for multiple testing ($P = 0.028$, $\alpha = 0.025$). We failed to observe an interaction between SNP3 and SNP8 having a significant effect on CD in the AA case–control sample.

Controlling for admixture effects on the association analysis in the above EA and AA case–control samples

using the program STRAT did not change the results substantially (data not shown), suggesting that admixture effects in our samples did not contribute to the observed associations.

DISCUSSION

In this study, the interaction between two independent *CNR1* variants, ie, SNP3^{^G⁺} and SNP8^{^T/T}, was associated with CD in an EA family sample. The association was replicable in an EA case–control sample. Sliding window haplotype analysis in the EA family sample showed that the haplotype constructed from SNP8 and its two neighboring SNPs displayed a higher association signal ($P = 0.007$) than

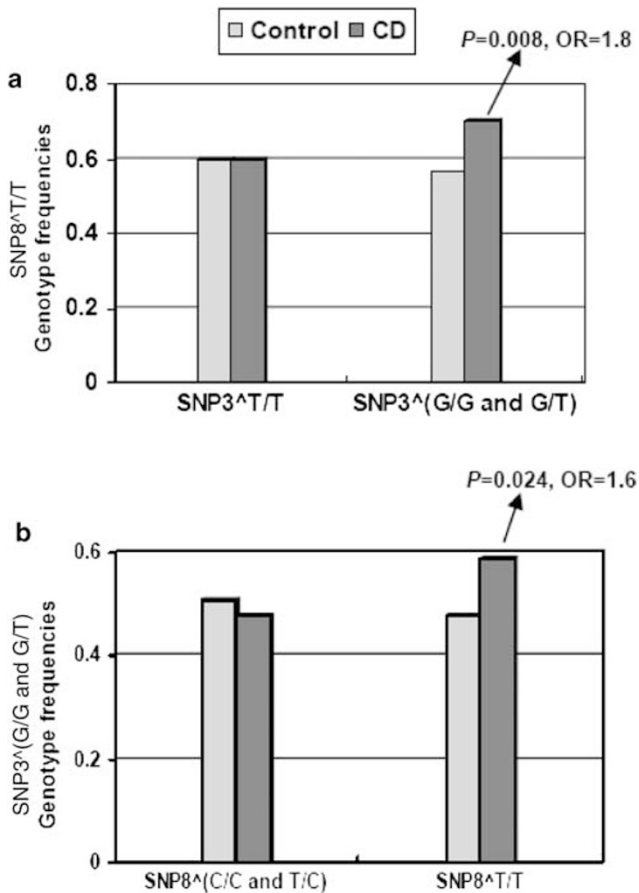


Figure 4 (a) SNP8^{T/T} genotype frequency difference between cocaine dependence (CD) cases and controls in different genotype groups of SNP3 in EAs. SNP3^{T/T} and SNP3^(G/G and G/T) represent T/T genotype and G allele-containing genotype of SNP3, respectively. (b) SNP3^(G/G and G/T) genotype frequency difference between CD cases and controls in different genotype groups of SNP8 in EAs. SNP8^(C/C and T/C) and SNP8^{T/T} represent C allele containing genotype and T/T genotype of SNP8, respectively.

SNP8 alone, suggesting that SNP8 is in LD with a disease-influencing locus rather than being a risk locus itself. This SNP8-linked risk locus could be located either in the coding region of *CNR1* directly affecting the function of the CB1 protein or in the 3'-UTR regulating translational efficiency, mRNA stability, or polyadenylation signals. Prediction of miRNA target sites for *CNR1* suggests that the 3'-UTR region of *CNR1* might be targeted by miRNAs affecting posttranscriptional *CNR1* expression (<http://www.targetscan.org>). SNP8 was in complete LD with a well-studied polymorphism in *CNR1*, ie, SNP7. This SNP is the Thr453Thr synonymous variant, located in the coding region of *CNR1*. Although it did not show association with CD, it showed nominal association with CIP. This SNP was reported to be associated with alcohol withdrawal delirium in an unrelated German population (Schmidt *et al.*, 2002).

Within the subjects with SNP8^{T/T}, we observed that SNP3^{G⁺} nominally significantly increased the risk for CD ($P=0.026$). Although this relationship fails rigorous criteria for statistical significance after correcting for multiple tests, the association may not be a random false positive. Within

the SNP3-containing haplotype block (Figure 1), a SNP, rs806380, which is correlated with SNP3 ($r^2=0.63$), was reported to be associated with cannabis dependence symptoms in an adolescent Caucasian sample (Hopfer *et al.*, 2006); a TAG haplotype in the SNP3-containing haplotype block was reported to be associated with polysubstance abuse in an EA sample (Zhang *et al.*, 2004). Further, another SNP in this SNP3-containing haplotype block, ie, rs2023239, was reported to be associated with CB1 binding in the prefrontal cortex (Hutchison *et al.*, 2008), supporting that this SNP3 locus may play a role in substance dependence. SNP3 *per se* could alter RNA secondary structure (IDT SciTools: <http://www.idtdna.com/SciTools/SciTools.aspx>). RAVEN (regulatory analysis of variation in enhancers (Andersen *et al.*, 2008)) predicts that SNP3 is located within a conserved region (phastCon score > 0.4) and may be a binding site for transcription factors and thereby influence the efficiency of *CNR1* transcription.

SNP3 and SNP8 are located within the same gene rather than two different genes, so it could be argued that the interaction effect detected in this study might simply reflect a haplotype effect which implicates a single risk variant effect for CD. But, as noted previously, SNP3 and SNP8 are neither in strong LD ($D' = 0.026$) nor correlated with each other ($r^2 = 0$); both SNP3 and SNP8 may have functional implication, suggesting that the interaction is more likely to imply joint effects of two independent and functional variants at the locations of SNP3 and SNP8.

The associations observed in the present study seem unlikely to be spurious for a number of reasons. First, as noted above, the association signals were replicable between case-control and family based samples. Second, the association signals of SNP3 and SNP8 were observed across five phenotype groups that were defined based on comorbidity with CD, ie, total CD, CDnoAD, CDnoOD, CDnoMD, and CDnoR-SD (Figure 3). The consistency across these five groups not only argues against a false positive association but also suggested that the association signals were from CD rather than from other types of SD. Third, another CD-related trait, CIP, also showed association with SNP8 in haplotype-wise analysis ($P_{\text{global}} = 0.003$), providing further evidence for an association between *CNR1* and CD. Fourth, the association signals were consistent when we used different analytic approaches. Genotype frequency comparison analysis showed that the genotype frequency of SNP8^{T/T} in CD cases was significantly higher than in controls among subjects carrying SNP3^G; logistic regression analysis showed that the interaction between SNP3^{G⁺} and SNP8^{T/T} significantly increased the risk for CD; and haplotype-wise analysis also consistently showed that the GT haplotype (constructed from SNP3^G and SNP8^T) increased risk for CD ($P=0.018$). Finally, as shown in Figure 2, both SNP8 and an SNP8-linked SNP, ie, SNP6, showed nominally significant association signals, but the other SNPs did not, which also argues against a false positive association at the SNP8 locus.

There is considerable evidence for both shared and nonshared genetic factors between different forms of substance dependence. Our initial study tested case-control-based association between *CNR1* and DD, which categorized CD cases and OD cases together, and AD was

also included in that initial study, but the specific association between *CNR1* and CD ($n_{EA} = 175$, $n_{AA} = 136$) was not analyzed previously (Zuo *et al*, 2007). This study greatly increased the sample size of CD cases ($n_{EA} = 299$, $n_{AA} = 406$) and analyzed the specific association between *CNR1* and CD. In this study, we also tested for family-based associations between *CNR1* and CD in two independent large samples, ie, an EA family sample ($n = 734$) and an AA family sample ($n = 834$). This study extended the initial study and had different study targets of interest.

There is only one prior study directly reporting an association between CD and an (AAT)_n polymorphism in *CNR1*, a population-based association study conducted in a male African-Caribbean sample (Ballon *et al*, 2006). African Caribbeans are an admixed population (Benn-Torres *et al*, 2008) and allele frequencies of *CNR1* variants differ among different ancestral populations (Covault *et al*, 2001; Herman *et al*, 2006; Zhang *et al*, 2004). Although an adjustment for admixture effects is required to avoid potential spurious associations, it was not included in that study. The (AAT)_n polymorphism is located between SNP9 and SNP10, 7 kb away from SNP10. SNP10 showed nominal association with CIP in the EA family sample in the present study. Thus, further work is warranted to investigate the association between this locus and CD.

CD is a complex non-Mendelian trait. This study shows that the positive likelihood ratio (LR^+) value for the subjects carrying SNP8^{A/T/T} to develop CD was 1.1, which shows that this variant of *CNR1* exerts a minor effect on the vulnerability to CD. This could explain why single locus analysis of this SNP in the case-control sample did not show a significant association with CD. Alternatively, the interaction model may more accurately reflect the genetic effects underpinning CD. The present study showed that the LR^+ value for interaction between SNP3^{A/G⁺} and SNP8^{A/T/T} was 1.4, considerably higher than that for either SNP3^{A/G⁺} or SNP8^{A/T/T} alone, showing that the interaction analysis is more powerful than the single locus analysis in detecting the association of *CNR1* with CD.

Whether or not CD is included, substance abuse and/or dependence have been reported to be associated with *CNR1* in some (Comings *et al*, 1997; Ponce *et al*, 2003; Schmidt *et al*, 2002; Zhang *et al*, 2004), but not all studies (Covault *et al*, 2001; Heller *et al*, 2001; Herman *et al*, 2006; Li *et al*, 2000). There are several potential explanations for these results. First, the inconsistent findings may result in part from a lack of phenotypic comparability across different studies. Substance dependence (SD) is a complex phenotype, which includes CD, OD, MD, AD, and others. This phenotypic heterogeneity could lead to different results. Second, inconsistent findings in relation to the association of *CNR1* and substance use disorders may also result from differences in the populations studied, as well as from population stratification and admixture effects. Most previous studies based population membership on self-report, which may not be adequate to exclude potential population stratification effects. Third, some of the differences could derive from the use of different marker sets and analytic models. Finally, the inconsistent findings could have resulted from small sample sizes leading to inadequate statistical power in some studies.

In addition to the above positive findings in EAs, we observed that SNP8^{A/T/T} conferred significantly higher risk for CD in the AA family sample, which is consistent with the finding in EA samples. In AA case-control sample, we failed to observe any significant association for SNP3 and SNP8; we also failed to detect a significant role of the interaction between SNP3^{A/G⁺} and SNP8^{A/T/T} on CD in AAs. The decay of LD caused by more recombination events that have occurred in AAs could underlie this finding (Figure 1). On the other hand, both the two SNPs showed HWD in cases and HWE in controls suggesting potential associations with CD in AAs too. A SNP8-containing haplotype (constructed from SNP8, SNP7, and a SNP between them) was reported to be associated with polysubstance abuse in AAs in another study (Zhang *et al*, 2004), supporting a role for SNP8 in CD in AAs.

In conclusion, this study provides evidence in support of an interaction model of the role of *CNR1* in the risk for CD in EAs. SNP8 locus may be implicated in CIP as well. *CNR1* could also be involved in the risk for CD in AAs. The two loci could ultimately be relevant to research on the prevention and treatment of CD. Further studies are warranted to replicate the findings in the present study, to locate the causal variants around SNP3 and SNP8 that contribute to risk of CD and to uncover the mechanism by which these two independent loci modulate that risk.

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DISCLOSURE/CONFLICT OF INTEREST

Dr Kranzler has received financial support or compensation for the following: related to consultation on grant reviews for the National Institutes of Health and related to academic lectures and editorial functions in various scientific venues; and has been a paid consultant to Alkermes Inc., Ortho-McNeil Pharmaceuticals, Thomson Healthcare, Sanofi-Aventis, Lundbeck, Forest Pharmaceuticals, Elbion NV, Bristol-Myers Squibb Co., and Solvay Pharmaceuticals. He has received research support from Ortho-McNeil Pharmaceuticals and Bristol-Myers Squibb Co. Dr Weiss has received financial support or compensation for the following: related to consultation on grant reviews for the National Institutes of Health and related to academic

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