CNR1 Variation Modulates Risk for Drug and Alcohol Dependence

Lingjun Zuo, Henry R. Kranzler, Xingguang Luo, Jonathan Covault, and Joel Gelernter

Background: Human cannabinoid receptor 1 (CB1), which is encoded by the *CNR1* gene, may play a role in the development of substance dependence (SD). Following initial reports of association of *CNR1* with SD, we studied multiple markers at this locus in a large case–control sample.

Methods: Ten *CNR1* markers and 38 ancestry-informative markers were genotyped in 451 healthy control subjects and 550 SD (AD and/or DD) patients (including European Americans [EAs] and African Americans [AAs]). Common confounding effects on association analysis of population stratification and admixture, age, and sex were controlled for using regression analysis. Disease risk and protective alleles were fine-mapped using a linkage disequilibrium measure (δ).

Results: In EAs, risk for each SD subtype significantly increased with the number of "G" alleles at rs6454674 (single nucleotide polymorphisms [SNP]3). SNP3^G^+ (the genotypes containing a G allele) and SNP8^T/T genotypes had significant interaction effects ($p = .0003$ for comorbid DD and AD, .0002 for DD, and .007 for AD). SNP3 and SNP8 together exerted stronger genetic effects on SD than either did individually. The peak δ values among all the markers were seen for SNP3 and SNP8 (rs806368).

Conclusions: We demonstrate that *CNR1* variation and interactive effects play important roles in risk for both DD and AD.

Key Words: Alcohol dependence, *CNR1*, drug dependence, fine-mapping, gene–gene interaction, substance dependence

Substance dependence (SD), including drug dependence (DD) and alcohol dependence (AD), is described as a cycle of increasing dysregulation of brain reward systems (Koob *et al.* 1997). The neurobiological mechanism for SD reward has been related to the mesocorticolimbic dopamine (DA) reward circuits (Blum 1996; Koob 1992; Koob *et al.* 1988, 2005; Le Moal and Simon 1991; Pontieri *et al.* 1996; Wise and Rompre 1989).

Several studies have shown that the endocannabinoid system serves to regulate DA reward circuits, an effect that may play an important role in the reward processes involved in SD (e.g., Giuffrida 1999; Manzanares 1999). The administration of delta-9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive ingredient of cannabis, increases extracellular DA concentrations in the nucleus accumbens (NAc), and this Δ^9-THC effect can be blocked by the μ opioid receptor antagonist naloxonazine when it is infused into the ventral tegmental area (VTA) (Chen *et al.* 1990; Tanda 1997). The endocannabinoid system also interacts with GABAergic and glutamatergic systems in the DA reward circuits (Mailleux and Vanderhaeghen 1994; Sanudo-Pena 1999; Sieradzan, 2001). Additionally, animal studies have demonstrated that both acute alcohol- and morphine-induced DA release in the NAc and dependence-inducing properties of opiates, cocaine, and alcohol were reduced in CB1 knockout mice, and similar results were obtained after the blockade of CB1 receptors with the selective CB1 receptor antagonist, SR141716A (Chaperon 1998; Cossu *et al.* 2001; Hungund *et al.* 2003; Ledent *et al.* 1999; Mascia *et al.* 1999; Tsuneyuke, 2000).

So far, two subtypes of cannabinoid receptors have been identified—the brain cannabinoid receptor (CB1) and the peripheral cannabinoid receptor (CB2) (Devane *et al.* 1988; Matsuda *et al.* 1990). CB1 is distributed widely throughout the central nervous system, mainly in the neocortex, hippocampus, basal ganglia, and cerebellum (Alger 2002; Herkenham 1992; Howlett 1990; Matsuda *et al.* 1993; Tsou *et al.* 1998). CB2 is distributed mainly in the immune system (Gurwitz and Kloog 1998). CB1 is a G-protein coupled receptor in presynaptic nerve terminals and is the target of Δ^9-THC.

CB1 is encoded by the cannabinoid receptor 1 gene (*CNR1*), which maps to 6q14–q15. Alternative splicing leads to two transcript variants, that is, transcript variant 1 (NM_016083/X81120), which encodes isoform A, and transcript variant 2 (NM_033181/X81121), which encodes isoform B. Recently, Zhang *et al.* (2004) found that a *CNR1* TAG haplotype consisting of rs806379, rs1535255, and rs2023293 was related to polysubstance abuse in both EAs ($p = 3.0 \times 10^{-5}$) and AAs ($p = .007$). They also found it to be significantly associated with AD in a Japanese sample ($p = 8.0 \times 10^{-6}$). *CNR1* seems to be a promising candidate gene for SD, but positive associations between *CNR1* and SD (including Comings *et al.* 1997) have not been confirmed by other studies (Covault *et al.* 2001; Heller *et al.* 2001; Herman *et al.* 2006; Li *et al.* 2000).

We conducted a population-based association study to investigate the role of *CNR1* in risk for DD (cocaine dependence [CD] and/or opioid dependence [OD]) and AD in EAs and AAs using a powerful study design. First, we considered confounders that are common in population-based association studies, such as population admixture, age, and sex, but that have generally not been considered in previous studies. Several studies have shown that the allele frequency distributions of many genetic variants at *CNR1* differ significantly by population and sex (Covault 2001; Herman *et al.* 2006; Li *et al.* 2000; Zhang *et al.* 2004). Furthermore, to varying degrees, AAs and EAs are admixed populations, that is, AA individuals often have some degree of European ancestry and EA individuals may have small proportions of African ancestry (Parra *et al.* 1998). Younger control subjects

From the Department of Psychiatry (LZ, XL, JG) and Genetics and Neurobiology (JG), Yale University School of Medicine, New Haven; VA Connecticut Healthcare System (LZ, XL, JG), West Haven Campus; and Alcohol Research Center (HRK, JC), Department of Psychiatry, University of Connecticut School of Medicine, Farmington, Connecticut.

Address reprint requests to Joel Gelernter, M.D., Yale University School of Medicine, VA Psychiatry 116A2, 950 Campbell Avenue, West Haven, CT 06516; E-mail: joel.gelernter@yale.edu.

Received October 16, 2006; revised November 27, 2006; accepted December 1, 2006.

0006-3223/07/$32.00
doi:10.1016/j.biopsych.2006.12.004

who are assessed as "healthy" may still develop SD when they become older. Thus, ethnicity, population admixture, sex, and age may confound gene-phenotype association analysis. Consequently, in this study, we performed structured association (SA) analysis and multivariate logistic regression analysis to control for these potential confounding effects. Second, we used a set of markers covering the full length of *CNR1*. Except for the studies by Zhang *et al.* (2004) and Herman *et al.* (2006), previous studies have focused on two *CNR1* polymorphisms, that is, the (AAT)n polymorphism and a 1359G/A variant at codon 453. The small number of markers studied and the incomplete linkage disequilibrium (LD) across the gene would be expected to limit the detection of association. Additionally, besides the known *CNR1* region shown in the current National Center for Biotechnology Information (NCBI) database, unknown functional variants in the 5′ flanking region of *CNR1* could affect disease risk. On this basis, we extended our region of interest to 67.09 kb across the *CNR1* locus and investigated 10 polymorphisms in this region based on the LD and fine-mapping information available from the current NCBI, ABI, and HapMap databases and the extant literature. Third, we considered multiple phenotypes that could be influenced to varying degrees by *CNR1* polymorphism. We also investigated the phenotype of comorbid DD and AD (DD&AD). Finally, single-locus analysis used in most previous studies simply reflects the association between certain markers and phenotype; limited LD might limit the ability to map the disease locus. To address these issues, we applied multivariate logistic regression analysis, haplotype trend regression (HTR; Zaykin *et al.* 2002), diplotype trend regression (DTR; Luo *et al.* 2006b), and marker–marker interaction analysis, which preserve much more genetic information than conventional single-locus analysis.

Methods and Materials

Subjects

Included in the study were 1001 subjects, comprising 451 healthy control subjects and 550 cases with CD, OD, and/or AD. This sample included two populations: 794 self-reported European Americans (EAs) and 207 self-reported African Americans (AAs). The ages were 28.5 ± 17.0 years for the control subjects and 39.5 ± 18.0 years for the cases. The control group consisted of 187 men and 264 women; in the cases, there were 405 men and 145 women. The cases met lifetime DSM-III-R or DSM-IV criteria (American Psychiatric Association 1987, 1994) for CD, OD, AD, or combinations of these disorders. The control subjects were screened to exclude major Axis I disorders, including SD, schizophrenia, mood disorders, and major anxiety disorders, using the Structured Clinical Interview for DSM-III-R, the Computerized Diagnostic Interview Schedule for DSM-III-R (C-DIS-R), the Schedule for Affective Disorders and Schizophrenia (SADS) (Spitzer and Endicott 1975), or an unstructured interview. The subjects were recruited at the University of Connecticut Health Center (UCHC) or the VA Connecticut Healthcare System—West Haven Campus. Genotyping data for rs806379 (single nucleotide polymorphisms [SNP]4) in an overlapping set of UCHC subjects (n = 817) were obtained independently and analyzed as part of another study (Herman *et al.* 2006); there was 100% concordance between duplicate genotypes in this overlapping set of subjects. All subjects gave informed consent before participating in the study, which was approved by the institutional review boards at each institution.

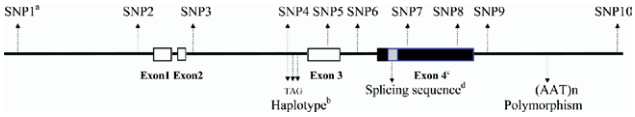

Figure 1. *CNR1* gene model and the genotyped markers. [a] Single nucleotide polymorphisms (SNP) numbers correspond to Table 1. [b] TAG Haplotype refers to that risk haplotype in the study by Zhang *et al.* (2004): T(rs806379), A(rs1535255), G(rs 2023293). [c] The exon 4 region corresponds to the exonic region of *CNR1* in National Center for Biotechnology Information build 36.2 and the University of California at Santa Cruz Browser. The other three exons in this figure are the potential exons proposed by Zhang *et al.* (2004). [d] The gray box refers to the splicing sequence between two variants for *CNR1*, variant 1 (NM_016083) and variant 2 (NM_033181).

Marker Selection and Genotyping

Ten *CNR1* SNP markers (average spacing, 7.45 kb) were selected. The 10 SNPs were designated as SNPs 1–10 in 5′ to 3′ order (Figure 1).

To detect the population structure of our sample, we genotyped 38 ancestry-informative markers (AIMs), including 37 short tandem repeat markers (STRs) and one Duffy antigen gene (*FY*) marker (rs2814778). The characteristics of this marker set have been described in detail previously (Yang *et al.* 2005).

All *CNR1* markers and the *FY* marker were genotyped using a fluorogenic 5′ nuclease assay method, the TaqMan technique (Shi *et al.* 1999). The 37 ancestry-informative STR markers were genotyped using the ABI PRISM 3100 semiautomated capillary fluorescence analyzer. Genomic DNA was extracted from peripheral blood by standard methods. Polymerase chain reaction conditions were described in detail elsewhere (Luo *et al.* 2005; Yang *et al.* 2005).

Statistical Analysis

Estimated ancestry proportion scores for each subject and number of ancestral populations were obtained through use of the program STRUCTURE, based on a model-based clustering method (Falush *et al.* 2003; Pritchard *et al.* 2000a). We set burn-in period length as 100,000, then used 100,000 Markov Chain Monte Carlo (MCMC) repetitions to obtain parameter estimates: ln Pr(X|K), where Pr denotes posterior probability, X denotes genotypes of the sampled individuals, and K denotes the number of populations assumed. The optimal K is considered to be the one with the highest posterior probability.

The correction for multiple tests was performed with the program SNPSpD to calculate the effective independent marker number from the nonindependent markers (Nyholt 2004).

The D′ values for each pair of *CNR1* markers were calculated and visualized through the program Haploview 3.0 (Barrett *et al.* 2005). Hardy–Weinberg Equilibrium (HWE) for the genotype frequency distribution of each marker was tested using the program PowerMarker (Liu and Muse 2004).

The case–control comparisons for allele and genotype frequency distributions were performed with the exact tests implemented in the program PowerMarker.

Structured association (SA) analysis was performed using the program STRAT (Pritchard *et al.* 2000b) to control for population admixture effects. We also performed multivariate logistic regression analysis in SPSS 14.0 to control for the other potential confounding effects. Because we do not know the exact genetic mode of inheritance for each marker beforehand, we performed this analysis under the genotype model ("model-free") first. Then we performed this analysis under the additive and/or recessive genetic models, which were indicated post hoc by our results. In the regression model, phenotypes served as the dependent variable,