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Association study of the *CNR1* gene exon 3 alternative promoter region polymorphisms and substance dependence

Aryeh I. Herman, B.A.[1], **Henry R. Kranzler, M.D.**[1], **Joseph F. Cubells, M.D., Ph.D.**[2], **Joel Gelernter, M.D.**[3], and **Jonathan Covault, M.D, Ph.D.**[1,4]

[1] Department of Psychiatry, University of Connecticut School of Medicine, Farmington, CT 06030, USA

[2] Department of Human Genetics, Emory University School of Medicine, Atlanta, GA

[3] Department of Psychiatry, Yale University School of Medicine, VA Connecticut Healthcare Center, West Haven, CT

Abstract

An alternative promoter producing a novel 5′-untranslated region of cannabinoid receptor mRNA has recently been described in *CNR1*, the gene encoding the cannabinoid receptor protein. Single nucleotide polymorphisms (SNPs) adjacent to this site were reported to be associated with polysubstance abuse (Zhang et al. 2004). We examined the association of 4 SNPs (rs6928499, rs806379, rs1535255, rs2023239) in the distal region of intron 2 of *CNR1* both with individual substance dependence diagnoses (i.e., alcohol, cocaine, and opioids), as well as with polysubstance dependence. The study samples consisted of European American and African American subjects with drug and or alcohol dependence (n=895), and controls (n = 472). Subjects were grouped as polysubstance dependent, opioid dependent, cocaine dependent, cannabis dependent and alcohol dependent. There was a modest association of marker rs1535255 with alcohol dependence, respectively (P=0.04), though with correction for multiple phenotype comparisons, this effect was not considered statistically significant. These findings fail to replicate the original report of an association between SNPs adjacent to an alternative *CNR1* exon 3 transcription start site and polysubstance abuse.

Keywords

CB1; genetic polymorphism; cannabinoid receptor

Introduction

Cannabis sativa preparations, the main psychoactive ingredient of which is Δ9-THC, produce intoxication characterized by sedation, cognitive dysfunction, failure to consolidate short-term memory, alteration in time assessment, perceptual changes, motor incoordination and poor executive function (Abood and Martin 1992; Dewey 1986; Hollister 1986; Pertwee 1988). In addition to exogenous cannabinoids, endocannabinoids including andamide and noladin ether, have also been identified. Neuropharmacologic effects of Δ9-THC, andamide, and noladin ether are mediated by the central cannabinoid receptor, CB1 (MIM 114610), encoded by *CNR1*, which maps to chromosome 6q14-q15 (Hoehe et al. 1991).

[4]To whom correspondence should be addressed at: Department of Psychiatry, MC 1410, 263 Farmington Avenue, Farmington, CT 06030-1410, Phone: 860-679-7560, Fax: 860-679-1316, E-mail: covault@psychiatry.uchc.edu.

The CB1 receptor is a G_i/G_o-coupled receptor abundant in brain regions important for drug reward and drug 'memories,' including the hippocampus, striatum, and cerebral cortex. The first polymorphism described in relation to *CNR1*(Dawson 1995) and the one that has been most widely studied is the $(AAT)_n$ trinucleotide short tandem repeat located 18,000 bp 3′ to the gene. Using a case-control design, Comings et al. (1997) reported an excess frequency of long $(AAT)_n$ repeats in a group of drug-dependent non-Hispanic Caucasians from Southern California (Comings et al. 1997). Other groups have been unable to replicate this finding (Covault et al. 2001; Heller et al. 2001; Li et al. 2000; Zhang et al. 2004).

A recent study reported a detailed molecular examination of the human *CNR1* locus and its variants (Zhang et al. 2004), which identified novel splice and promoter variants that give rise to additional exons encoding alternative 5′UTRs. One alternate 5′UTR results from activity of a secondary promoter within intron 2, which displayed regionally selective expression in brain. The report also included examination of the association with polysubstance abuse of 19 markers that extend 30,000 bp and encompass all of *CNR1*. Three SNPs (rs806379, rs1535255 and rs2023239) in intron 2, adjacent to the exon 3 alternate transcription initiation site, as well as the haplotype including the minor allele at each of these SNPs, were associated with polysubstance abuse in both European-American (EA) (n=526) and African-American (AA) (n=311) samples. We sought to replicate the association of these *CNR1* markers with polysubstance dependence. Additionally, we examined whether the 3-SNP haplotype is associated with alcohol dependence in the absence of drug dependence, as was suggested in a study of a sample of Japanese alcoholics that was included in the report by Zhang et al. (2004).

Materials and Methods

Subjects

Subjects were recruited as part of ongoing studies of the genetics of substance use disorders or from clinical trials for the treatment of alcohol dependence at the University of Connecticut Health Center (UCHC), Farmington, CT and VA Connecticut Healthcare Center (VACT), West Haven, CT. Control subjects were recruited by advertisement in the greater Hartford, Connecticut area. Subjects previously examined at the CNR1 (AAT)n marker by our group (Covault et al. 2001) were included in the current sample and represent 38% of the 1,367 subjects examined here. Psychiatric diagnoses were made using the Structured Clinical Interview for DSM-III-R or DSM-IV (SCID) (First et al. 1997) or the Semi-Structured Assessment for Drug Dependence and Alcoholism (Gelernter et al. 2005; Pierucci-Lagha et al. 2005). Substance dependent subjects with a lifetime diagnosis of schizophrenia were excluded. All control subjects were screened using the SCID or the SSADDA to exclude individuals with a diagnosis of substance abuse or dependence. Subjects provided written, informed consent to participate in study protocols approved by the institutional review boards at the UCHC, Yale University School of Medicine, or VACT, and were paid for their participation.

Genotyping

DNA was extracted from whole blood using the PureGene kit (Gentra, Minneapolis, MN) or standard salting out methods. SNPs were genotyped using TaqMan™ 5′-nuclease assay methods (Livak et al. 1995; Shi et al. 1999) together with an ABI 7500 Sequence Detector instrument (Applied Biosystems, Foster City, CA) using probes containing the non-fluorescent minor groove binding 3′-quencher MGB (Applied Biosystems). Primer and probe sequences described by Zhang et al. (2004) were used for markers rs806379, rs1535255 and rs2023239 (Table I). The reverse primer for the rs806379 SNP was redesigned by displacing the 3′-end by 10 nucleotides to avoid inclusion of the rs6928499 G/

C SNP. The rs806379 assay primer used by Zhang et al. has the rs6928499 G-allele (common) at the 6th nucleotide from the 3′ end of the primer. In initial genotyping we observed indistinct clusters for A/T heterozygotes, which limited our ability to reliably distinguish A/T from A/A clusters using the rs806379 primers described by Zhang et al. This was presumably related to different priming efficiencies related to the presence of the rs6928499 SNP within the reverse primer binding site. Primers and probes for the SNP rs6928499 were designed using the ABI Primer Express software. Ten ul PCR reactions contained 10 ng genomic DNA, 500 nM each primer, 100–160 nM for each probe, and 1X ABI TaqMan Master Mix. Samples were theromocycled 40 times for 15 sec at 94°C and 60 sec at 60°C (58C for rs6928499 and rs2023229) and change in fluorescence quantitated by comparison of pre- and post-PCR readings. At least 10% of samples were repeated for each SNP with genotyping error rates of <0.02.

Data analysis

Diagnostic groups were compared on age using ANOVA and on sex using a 2 X 2 contingency table and the χ^2 test. Age and sex were compared as functions of genotype using ANOVA and the χ^2 test using 2 × 3 contingency tables, respectively. The control and substance dependence groups were compared on allele frequencies in 2 X 2 contingency tables using the χ^2 test. Multinomial linear regression analysis was used to test for differences in SNP genotype frequency resulting from the interaction of sex and substance dependence. Haplotype and linkage disequilibrium analysis was conducted using Haploview 3.2 (Barrett et al. 2005). We report raw χ^2 test significance values, but note that correction for multiple phenotype comparison groups (n=5) would require a Bonnferoni corrected p=0.01 for statistical significance.

Results

Thirteen hundred sixty seven unrelated subjects including 895 subjects with drug and/or alcohol dependence [615 European American (EA) and 280 African American (AA)] and 472 control subjects (388 EA and 84 AA) were genotyped using the TaqMan 5′ nuclease assay at 5 SNPs encompassing 5,000 bp in the 5′ flanking region of the alternative exon 3 *CNR1* transcript initiation site (see Table I for marker position relative to exon 3 transcript initiation site). All markers examined showed high LD between adjacent markers in both EA and AA subjects (D′ =0.95–0.98).

Genotypes at all markers were in Hardy-Weinberg equilibrium for EA subjects (p= 0.55–0.84). For AA subjects, genotypes for SNP rs806379 were not in Hardy-Weinberg equilibrium (p=0.03), with a lower than expected number of heterozygotes, while genotypes for the other 3 SNPs were in Hardy-Weinberg equilibrium (p= 0.54–0.99).

Allele frequencies were compared between control subjects and groups of subjects dependent on cocaine, opioids, cannabis or with polysubstance dependence (i.e., those dependent on 2 or more of the following drugs: alcohol, cocaine, opioids or cannabis). Additionally, since EA drug dependent subjects in our sample had a high prevalence of co-morbid alcohol dependence, we compared a group of 214 EA subjects with dependence on alcohol but no other drugs. Demographic and diagnostic features of the groups are shown in Tables II and III. Substance dependence subjects were on average older (EA-39.1 ± 9.5 vs. 28.3 ± 8.4 yo, p<0.001; AA-38.9 ± 7.6 vs. 31.9 ± 10.0 yo, p<0.001) and more likely to be male (EA-71% vs. 34%, p<0.001; AA-65% vs. 35%, p<0.001) compared with control subjects. There was no association of genotype for any of the 4 SNPs with age (EA subjects p=0.29–0.99 and AA subjects p=0.09–0.97) or sex (EA subjects p=0.09–0.98 and AA subjects p=0.30–0.91).

Allele frequencies for control and drug dependent groups are shown in Tables IV and V. We observed a significant difference in allele frequency between EA and AA subjects for markers rs6928499, rs1535255, and rs2023239 (χ^2=67–84, $p<10^{-15}$), with AA subjects having nearly twice the frequency of the minor allele at each of these markers. Allele frequencies for the rs806379 SNP were not significantly different between the two populations.

Allele frequencies differed significantly by phenotype for 1 SNP in the EA sample and none in the AA sample. Among EAs, there was an excess frequency of the minor allele for marker rs1535255 in alcohol dependent individuals (χ^2=4.1, nominal p=0.04). There was a non-significant excess frequency of the minor allele for the 3 adjacent markers flanking rs1535255 in alcoholics. There was no significant difference in the frequency of the 4-SNP haplotype comprised of these markers (0.203 vs. 0.169; χ^2=2.3, p=0.13) between alcoholic and control subjects. None of our observed allelic associations are considered statistically significant when controlled for multiple phenotype comparisons. Additionally, examination of potential sex effects on the association of substance dependence and genotype using multinomial regression analysis failed to demonstrate any interaction of sex on the frequency of marker genotypes for any of the substance dependence groups for either race (p>0.05). Haplotype frequencies for the 3 SNP haplotype examined by Zhang et al. (2004) are shown in Tables VI and VII. There were no statistically significant differences in haplotype frequencies between control and substance dependance groups.

Discussion

Zhang et al. (2004) reported that, compared with controls, the minor allelic frequency of markers rs806379 and rs2023239 was significantly higher in a sample of 351 EA polysubstance abuse subjects and that the minor allele frequency of rs806379, rs1535255, and rs2023239 was significantly higher in a sample of 235 AA polysubstance abuse subjects. Our results fail to replicate the association of these markers either with dependence on individual substances (i..e, cocaine, opioids, or cannabis) or polysubstance dependence. Although we noted an excess frequency of the rs1535255 minor allele in a sample of 214 EA alcohol dependent individuals without comorbid drug dependence (nominal p=0.04), correction for multiple testing rendered the association non-significant. There were no significant allele frequency differences between the substance dependence groups and controls in our AA sample.

There are a number of potential explanations for the different findings obtained by us compared with Zhang et al. (2004). Our sample included only individuals who met diagnostic criteria for substance dependence, while the sample studied by Zhang et al. were characterized as “polysubstance abusers.” Zhang et al. did not report information on the sex or substances abused by their study sample, nor did they specify the diagnostic criteria they employed, which makes it difficult to identify potential differences between the subjects studied by them and those included in the present report. Second, the primers that we used to detect SNP rs806379 were selected to avoid overlap with the adjacent SNP rs6928499, which may have contributed to differences in allele frequency for marker rs806379 between the two studies. Our EA sample similar in size (EA) as that studied by Zhang et al., which argues against inadequate statistical power as an explanation for our failure to replicate the previous findings in EAs. Our EA sample was large enough to yield >99% power at α=0.05 to detect a difference in frequency of the minor allele 3 SNP haplotype based on the effect reported by Zhang et al.. In contrast, our AA sample provided 64% power to detect the observed effect size reported previously for AA subjects.

There are limitations to our study. Most of our clinical sample was diagnosed as alcohol dependent. The predominance of alcohol dependence in our sample may not generalize to a substance dependent population that is predominantly drug dependent. Further, there are multiple SNPs that are located further 5′ within the *CNR1* intron 2 region, which we did not examine, so we would have failed to detect association with those polymorphisms. Finally, we note that haplotype frequencies reported here in tables V and VI are markedly different than those reported by Zhang et al. This is in large part due to the finding of the opposite minor allele base for markers rs806379 and rs1535255 in the two studies despite a similar minor allele freqeuncy in the two studies. We report the SNP variant bases per the NCBI refSNP marker database, which corresponds to the chromosome 6 plus strand in each case. Zhang et al. do not describe the convention they used in presenting SNP data but appear to use the base designation for the chromosome 6 minus strand for these markers (which is sense with respect to the CNR1 gene). With this difference in mind, we report the plus strand minor allele base for the markers rs806379, rs1535255 and rs2023239 as T, G and C vs. that in Zhang et al. of T, A and G.

This research was focused on replication of the original Zhang et al. (2004) report of an association between a *CNR1* 3 SNP haplotype and polysubstance abuse. We were unable to replicate the association of these markers either with polysubstance dependence or with dependence on individual substances (cocaine, opioid, cannabis, or alcohol). Further research is needed to determine whether allelic association exists between CNR1 and substance dependence.

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Table I

Primer and probe sequences for *CNR1* exon 3 transript inititation 5′ flanking SNPs with SNP postion relative to exon 3 alternate transcript initiation site.

SNP (Position)	Primers	MGB-Probe	[Probe]
rs6928499 (−407)	F-CCTAAATCGCAGAACTGATCTGAA R-GCAAAGAGCCATAATACTAAGTAATGATAA	VIC-ATGTAAAACATA**G**TGCCTGAC FAM-TAAAACATA**C**TGCCTGAC	160 nM VIC 120 nM FAM
rs806379 (−385)	F-AATGCCTAAATCGCAGAACTGATCT R-AATAATACCCATTGAAGACTTACTTTGTGTCA	VIC-ACATGCATTTAAT**A**TCATC FAM-TAACATGCATTTAAT**T**TCATC	100 nM each
rs1535255 (−326)	F-CTTGGGCAATCAGTCTTTCTAAGC R-AGATCAGTTCTGCGATTTAGGCATT	VIC-CTCATCCCC**C**TTTTAC FAM-CATCCCC**A**TTTTAC	100 nM each
rs2023239 (+400)	F-GAGTTGAAAGGCAAAAGCTAGGTTT R-GGGACACAGAAGACAGTCACAATAT	VIC-CTGTTCCTTAC**G**TGGTCC FAM-TGTTCCTTAC**A**TGGTCC	140 nM VIC 100 nM FAM

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Table II

Phenotypic features of non-Hispanic Caucasian study subjects.

	Control (n=388)	Cocaine dependence (n=340)	Opioid dependence (n=176)	Cannabis dependence (n=200)	Polysubstance dependence (n=350)	Alcohol dependence only (n=214)
% lifetime dependence						
alcohol	0	74	74	84	86	100
cocaine	0	100	82	76	85	0
opioid	0	43	100	50	48	0
cannabis	0	45	56	100	56	0
% male	34	69	62	70	67	77
age, mean (S.D)	28.3 (8.4)	36.8 (7.9)	36.3 (8.9)	35.1 (7.7)	36.6 (8.0)	43.8 (9.6)

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Table III

Phenotypic features of African American study subjects.

% lifetime dependence	Control (n=84)	Cocaine dependence (n=268)	Opioid dependence (n=101)	Cannabis dependence (n=92)	Polysubstance dependence (n=205)
alcohol	0	58	60	78	79
cocaine	0	100	86	97	97
opioid	0	32	100	43	45
cannabis	0	33	40	100	44
% male	35	65	61	65	63
age, mean (S.D)	31.9 (10.0)	39 (7.6)	41 (8.4)	38.5 (7.7)	39 (7.7)

Table IV

Minor allele frequencies in European American substance dependent subjects and controls

CNR1 SNP (major/minor allele 6q15+ strand)	Control (n=758[a])	Cocaine dependence (n=640)	Opioid dependence (n=334)	Cannabis dependence (n=344)	Polysubstance dependence (n=658)	Alcohol dependence only (n=390)
rs6928499 (G/C)	0.182	0.162	0.188	0.195	0.169	0.216
rs806379 (A/T)	0.479	0.465	0.535	0.488	0.488	0.500
rs1535255 (T/G)	0.172	0.164	0.189	0.185	0.167	0.223*
rs2023239 (T/C)	0.181	0.155	0.177	0.176	0.162	0.215

n=number of chromosomes successfully genotyped.

* p<0.05

Table V

Minor allele frequencies in African American substance dependent subjects and controls

CNR1 SNP (major/minor allele 6q15+ strand)	Control (n=138[a])	Cocaine dependence (n=490)	Opioid dependence (n=186)	Cannabis dependence (n=168)	Polysubstance dependence (n=394)
rs6928499 (G/C)	0.302	0.355	0.315	0.375	0.338
rs806379 (A/T)	0.538	0.517	0.516	0.537	0.519
rs1535255 (T/G)	0.336	0.355	0.316	0.374	0.342
rs2023239 (T/C)	0.306	0.333	0.293	0.339	0.314

[a]n=number of chromosomes successfully genotyped.

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Table VI

Three SNP haplotype frequencies in European American substance dependent subjects and controls.

Haplotype[a]	Control (n=758 [b])	Cocaine dependence (n=640)	Opioid dependence (n=334)	Cannabis dependence (n=344)	Polysubstance dependence (n=658)	Alcohol dependence only (n=390)
ATT	0.511	0.518	0.449	0.499	0.497	0.491
TTT	0.306	0.319	0.361	0.314	0.334	0.287
TGC	0.171	0.147	0.170	0.172	0.154	0.202
AGC	-	-	-	-	-	-

[a]-haplotypes defined by snps rs806379, rs1535255, rs2023239 with frequency >0.03 in EA or AA subjects.

[b]n=number of chromosomes successfully genotyped.

Table VII

Three SNP haplotype frequencies in African American substance dependent subjects and controls.

Haplotype[a]	Control (n=138[b])	Cocaine dependence (n=490)	Opioid dependence (n=186)	Cannabis dependence (n=168)	Polysubstance dependence (n=394)
ATT	0.426	0.464	0.444	0.447	0.462
TTT	0.233	0.175	0.241	0.175	0.192
TGC	0.277	0.308	0.259	0.322	0.291
AGC	0.033	0.017	0.039	0.012	0.017

[a]- haplotypes defined by snps rs806379, rs1535255, rs2023239 with frequency > 0.03.

[b]n=number of chromosomes successfully genotyped.

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