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Further evidence for association between genetic variants in the cannabinoid receptor 1 (*CNR1*) gene and cocaine dependence: Confirmation in an independent sample and meta-analysis

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Abstract

Genetic research on cocaine dependence may help clarify our understanding of the disorder as well as provide insights for effective treatment. Since endocannabinoid signaling and dopamine neurotransmission have been shown to be involved with drug reward, genes related to these systems are plausible candidates for susceptibility to cocaine dependence. The cannabinoid receptor 1 (*CB1*) protein regulates both the endocannabinoid and dopaminergic neurobiological systems, and polymorphisms in the cannabinoid receptor gene, *CNR1*, have been previously been associated with substance dependence. In this study, we attempt to replicate a finding associating *CNR1* with cocaine dependence in African Americans. Cocaine dependent individuals (n=883) and unaffected controls (n=334) of African descent were genotyped for two single nucleotide polymorphisms (SNPs) in the *CB1* gene (rs6454674, rs806368). We observed a significant difference in genotype frequencies between cases and controls for both SNPs (p = 0.05). This study confirms the association between variants in the *CNR1* and cocaine dependence. However, considering the substantial co-morbidity of cocaine dependence with other drugs of abuse, additional studies are necessary to determine whether polymorphisms in *CNR1* induce a general susceptibility to substance dependence or are specific to cocaine addiction.

Keywords

Addiction; Association study; Cocaine; Cannabinoid receptor; Genetics; Substance abuse

Introduction

The 2002 National Survey on Drug Use and Health revealed that nearly 6 million Americans age 12 years and above used cocaine during the year prior to the survey, making cocaine one

of the most commonly used illicit drugs in the United States (Nsdhu, 2003). Repeated exposure to cocaine can lead to the rapid escalation of compulsive drug seeking behavior, which persists despite negative socioeconomic and health consequences, and is characteristic of cocaine dependence. It has been estimated that 5-6% of recent cocaine users will become cocaine dependent after the onset of first use (O'Brien & Anthony, 2005). To date there are no Food and Drug Administration (FDA) approved pharmacological treatments for cocaine dependence and therefore studies into the genetic basis of this disorder may help elucidate the neurobiological basis of cocaine dependence and in turn generate new pharmacological therapies.

The neurotransmitter dopamine is known to play a major role in drug reward (Dackis & O'Brien, 2005). Elevations in extracellular dopamine (DA) in brain regions such as the nucleus accumbens and the ventral striatum are believed to underlie the reinforcing properties of cocaine (Di Chiara *et al.*, 2004, Volkow, 2002) as cocaine binds to DA receptors and decreases DA re-uptake (Hyman *et al.*, 2006). Cannabinoid 1 receptors (CB1) are G-protein coupled receptors which are densely expressed throughout the mesolimbic system (Herkenham *et al.*, 1990). CB1 plays a direct role in the dopaminergic reward pathway, relaying signal cascades at the terminals of striatal medium spiny neurons, regulating dopamine and glutamate release as well as the long-lasting neuroplastic desensitizing effects inherent with chronic cocaine use (Cheer *et al.*, 2007a, Corbillé *et al.*, 2007, Filip *et al.*, 2006, Xi *et al.*, 2006).

Pharmacological blockade of CB1 receptors has been shown to reduce the inhibitory effect of cocaine on GABA neurotransmission in rats (Centonze *et al.*, 2004) and attenuate the dopaminergic increases in the nucleus accumbens induced by cocaine administration (Cheer *et al.*, 2007b). CB1 receptors have also proven to be relevant for relapse into cocaine seeking behavior as CB1 antagonists (SR141716) administered during prolonged cocaine withdrawal have been shown to reduce cocaine seeking behaviour induced by cue-exposure. Conversely, pre-treatment with CB1 agonists increases relapse behavior in mice undergoing cocaine withdrawal (De Vries *et al.*, 2001).

While the interplay between genetic and environmental factors underlying cocaine dependence is not fully understood, studies have estimated that approximately two thirds of an individual's risk for developing cocaine dependence is heritable (Kendler *et al.*, 2000, Kendler & Prescott, 1998). As the cannabinoid system is known to influence dopaminergic signaling and relapse to cocaine seeking behavior in animals, genetic studies analysing polymorphisms in *CNR1* may reveal genetic variation that increases the risk for cocaine dependence. Two SNPs in *CNR1*, rs6454674 and rs806368, were found to be associated with cocaine dependence and cocaine induced paranoia across three independent populations of European and African Americans (Zuo *et al.*, 2009). These same SNPs were found to be associated with alcohol and illicit drug dependence in European Americans (Zuo *et al.*, 2007) and rs806368 has demonstrated association with cannabis dependence (Agrawal *et al.*, 2008). The purpose of the current study was to analyze these two SNPs in an independent African American population of cocaine dependents in order to provide replication of the findings of Zuo *et al.* (Zuo *et al.*, 2009). The two SNPs, rs6454674 and rs806368, were chosen for replication because of their consistent association with both cocaine and general substance dependence.

Materials and Methods

Sample collection

Blood samples for DNA isolation from cocaine-dependent individuals of African-American decent (n=926; 67% male, mean age: 43) were collected during clinical studies of cocaine

dependence at the University of Pennsylvania Treatment Research Center. Subjects were at least 18 years of age. All were assessed with the Structured Clinical Interview for DSM Disorders (SCID) and urine drug screens were obtained. All patients had a clinical diagnosis of cocaine dependence as defined by *DSM-IV*. Family history was not obtained and ethnicity was determined by self-report. All psychiatric axis I disorders except alcohol dependence/abuse and nicotine dependence were used as exclusion criteria. In addition, participants were excluded if they had a history of a seizure disorder (except cocaine-induced seizures) or a severe medical illness, including a history of AIDS (but not merely of HIV+ status). Individuals currently being treated with psychotropic medications or with psychiatric symptoms, including psychosis, dementia, suicidal or homicidal ideation, mania or depression requiring antidepressant therapy were also excluded. Blood samples from control persons of African-American descent (n=346; 32% male, mean age: 40) were collected at the University of Pennsylvania, Thomas Jefferson University, and the National Institute of Mental Health Genetics Initiative (www.nimhgenetics.org). Control individuals were screened for history of substance use disorders or other psychiatric illness. They were not assessed with a urine drug screen and ethnicity was determined by self-report. Subjects with a history of substance dependence or a history of major psychiatric illness were excluded from this study (Berrettini & Persico, 1996). Genomic DNA was extracted from peripheral leukocytes within obtained blood samples by standard protocols. All protocols were approved by the Institutional Review Boards at Thomas Jefferson University and the University of Pennsylvania, and all subjects provided written informed consent before blood sample collection.

SNP selection and genotyping

The *CBI* gene is located on chromosome 6q14-q15. *CBI* contains 1 exon and spans 26,084bp (Ensembl Human Exon View accession ENST00000369499). SNPs for genotyping were selected based on previous positive findings in a European American population (SNP1: rs6454674; SNP2: rs806368) (Zuo *et al.*, 2009). SNP genotyping was performed using Applied Biosystems Inc. (ABI) (Foster City, CA, USA) 'Assays-on-demand' as per manufacturer protocol.

Statistical analysis

The allelic and genotypic association of SNPs with cocaine dependence was determined using the chi-square test and p-values were corrected for multiple comparisons using the false discovery rate (FDR) procedure (Benjamini *et al.*, 2001) in the software package PLINK v1.04 (Purcell *et al.*, 2007). Correction for multiple testing was carried out using the false discovery rate (FDR) method (Benjamini & Hochberg, 2000).

Haplotype analysis was performed in PLINK using the expectation maximization (EM) algorithm implicated in the software packages (<http://pngu.mgh.harvard.edu/~purcell/plink/>) (Purcell *et al.*, 2007). Permutation analysis of the haplotypic associations was conducted to randomly reassign the case and control labels in the dataset and give a significance level corrected for all markers or haplotypes tested. A total of 10 000 permutations were performed in each permutation analysis.

Our sample size had reasonable power to detect a disease association at a *P*-value less than or equal to 0.05, assuming an odds ratio of 1.5 and a minor allele frequency of 30% (82% for a general 2df test, 89% for a dominant, and 15% for a recessive mode of inheritance).

Results

Measures of linkage disequilibrium (LD) between the two markers were shown to be weak ($r^2 = 0.002$, $D' = 0.179$) which is consistent with the findings of Zuo et al who also analysed these SNPs in an independent African American population (Zuo *et al.*, 2009). Quality control was maintained by genotyping 10% duplicates for cases and controls, and the concordance rate for our genotyping was calculated to be 99.92%. None of the genotype counts deviated significantly from Hardy-Weinberg equilibrium in the total population (Balding, 2006) however, rs806368 displayed a significant deviation from Hardy-Weinberg amongst the cases ($p=0.03$). State the nature of the deviation, eg, an excess of homozygotes for the minor allele.

Single marker analysis yielded evidence for genotypic association for both rs806368 ($p=0.017$, OR=0.75, 95% C.I =0.54-0.96) and rs6454674 ($p=0.05$, OR=1.14, 95% C.I=0.94-1.39). The C allele of rs806368 was found to have a protective effect against cocaine dependence and was observed at a higher genotype frequency in controls compared to cases. For rs6454674 the G allele was observed to be at a higher frequency in cases that were minor homozygotes for this allele (0.13 vs 0.08) and therefore conferred risk for cocaine dependence. Genotypic and allelic associations are summarized in table one. After performing genotypic associations it became apparent that rs6454674 was acting under a recessive mode of inheritance and therefore, performing a chi-square test to analyze the recessive SNP action of rs6454674 (DD vs Dd,dd) increased the significance to $p=0.017$. The genotypic tests for association for both SNPs withstood correction for multiple testing according to the false discovery method (FDR $P= 0.05$). Haplotype analysis did not show significant associations for two marker analysis (data not shown).

A meta-analysis was conducted by combining the genotype counts from the African American individuals reported in Zuo et al (Zuo *et al.*, 2009) with that of the current study. When the data was combined rs806368 no longer remained significant ($p=0.19$) however, rs6454674 increased in significance ($p=0.004$). These data are presented in table two.

Discussion

The data presented in this study demonstrate an association of 2 *CNR1* SNPs, rs6454674 and rs806368, with cocaine dependence in individuals of African descent. In addition, we performed a meta-analysis using the data reported by Zuo and colleagues (Zuo *et al.*, 2009). The meta-analysis provides the largest sample of African American cocaine dependents and controls ($N=1800$) studied for genetic risk for cocaine dependence to date, and the findings display an increased significance from $p=0.02$ in our sample to $p=0.004$ for rs6454674. These data confirm the relevance of these 2 polymorphisms when considering genetic risk for cocaine dependence in African Americans; however it should be noted that our findings may reflect an association with a more general substance abuse phenotype. Poly-substance abuse is high amongst cocaine dependents, it has been reported that amongst cocaine dependants alcohol co-dependence occurs approximately 85% of the time in the community (Regier *et al.*, 1990). rs6454674 has previously been associated with alcohol dependence and drug dependence (cocaine and opioids) in European Americans (Zuo *et al.*, 2007). Furthermore, rs806368 has been modestly associated with cannabis dependence in European Americans ($P=0.05$) and the co-morbidity with alcoholism in this sample was estimated to be roughly 70% (Agrawal *et al.*, 2009). As CB1 has been found to interact with brain dopaminergic, glutamatergic, GABAergic and opioidergic systems its association with substance dependence in general is plausible considering the receptor mediates the neurochemical effects of nearly all drugs of abuse (Ameri, 1999, Manzanares *et al.*, 1999, Romero *et al.*, 2002). The following sentence is incorrect and must be re-written.

Interestingly animal models have shown that CB1 does not modulate the primary reinforcing effect of psychostimulants as *Cnr1* null mice do not self-administer cocaine or show changes to conditioned place preference when compared to wild-types (Cossu *et al.*, 2001). However, CB1 is required for the maintenance of cocaine seeking behavior as *Cnr1* knockout mice will not continue to seek cocaine when the effort to obtain cocaine infusions is enhanced (Soria *et al.*, 2005). Conversely, mice lacking *Cnr1* show a decreased alcohol preference and a reduction in ethanol place preference (Hungund *et al.*, 2003, Thanos *et al.*, 2005, Wang *et al.*, 2003). Therefore, considering the high co-morbidity of alcohol dependence with cocaine and cannabis dependence, future work should be carried out to determine whether *CNR1* polymorphisms are relevant for addiction to cocaine or whether this reflects a stronger association with alcohol dependence.

One caveat to our finding is that there are very few homozygotes for the minor allele among the controls and the cases at rs806368, suggesting that the statistical significance may be a false positive. Larger sample sizes are required when the minor allele frequency is low. Our finding associating rs654657 with cocaine dependence in African Americans confirms the findings of Zuo *et al.* however rs806368 was only found to be associated with European Americans in this study as they report no association in African Americans. It should be noted that genetic studies including individuals of self-reported ancestry are susceptible to population stratification (Freedman *et al.*, 2004) and the genetic admixture in African Americans has been shown to be larger than expected (Parra *et al.*, 2001, Pfaff *et al.*, 2004, Tian *et al.*, 2006). Indeed, rs806363 has a higher minor allele frequency in Europeans ($C=0.19$) compared to Yoruban Africans in Nigeria ($C=0.08$) according to data from the International Hapmap Project (www.hapmap.org). Therefore, different degrees of population admixture between the two African American samples may be responsible for the discrepancies in the association analyses. There are other studies which have failed to associate *CNR1* with substance dependence however these studies have either had considerably smaller sample sizes (Heller *et al.*, 2001) or analysed different polymorphisms to the ones presented in this study (Covault *et al.*, 2001, Li *et al.*, 2000). Indeed, the effect sizes reported in this association study for each SNP are modest ($OR = 1.14$) and therefore a large sample size would be needed to detect such a small effect. Despite this, the result should be interpreted with caution and further replication may be needed to confirm the role of rs806363, if any, in cocaine dependence.

In order to understand the biological relevance of these findings it is important to know how these 2 polymorphisms in *CNR1* convey risk for cocaine dependence. rs806368 is located in the 3'UTR and is in a region of the genome characterized by high evolutionary conservation (Perhaps show a figure with the diagram of the gene and the location of the SNPs with the r^2 value. Also show the evolutionary conservation. It is plausible therefore that this SNP affects the stability of the *CNR1* mRNA transcript which may reduce the rate of translation. This may have biological consequences relevant for cocaine dependence. However, the 2 SNPs analyzed in this study may only be acting as proxies for the true causal variants and therefore additional work is needed to identify the mechanism by which these genetic variants increase the risk for cocaine dependence. In conclusion, there is now substantial emerging data to suggest that polymorphisms in *CNR1* are relevant for susceptibility to substance dependence. One group of individuals for whom this is becoming increasingly relevant is cocaine dependents of African descent. Further work is warranted to identify the biological mechanisms by which *CNR1* SNPs increase risk for cocaine dependence and also to determine whether *CNR1* is specifically relevant for cocaine dependence or substance dependence in general.

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Table one

Comparison of genotype and allele frequencies in 2 CNR1 SNPs. P-values represent PLINK generated χ^2 tests for association between cocaine dependent cases and unrelated controls.

SNP	Population (N)	AA	Aa	aa	Minor Allele	P-Value	MAF	P-Value	OR (95% C.I)
rs806368	Cases (883)	0.817	0.182	0.001	C	0.017 ¹	0.09	0.05	0.75 (0.54-0.96)
	Controls (334)	0.78	0.21	0.02			0.12		
rs6454674	Cases (859)	0.42	0.45	0.13	G	0.05 0.017 ²	0.35	0.19	1.14 (0.94-1.39)
	Controls (316)	0.43	0.49	0.08			0.32		

MAF = Minor Allele Frequency, OR = Odds ratio, 95% C.I = Confidence intervals

¹ Fishers exact P-value,

² P-value represents recessive test for association (AA +Aa vs aa). Bold highlighted p-values remain significant (p 0.05) after correction for multiple testing using the FDR method.

Meta-analysis comparing genotype frequencies in cocaine dependents and unrelated controls using data from the present study combined with genotype counts from the African Americans genotyped in Zuo et al (2009).

Table two

SNP	Population (N)	AA	Aa	aa	P-Value
rs806368	Cases (1276)	0.817	0.175	0.008	0.19
	Controls (543)	0.80	0.19	0.02	
rs6454674	Cases (1193)	0.44	0.42	0.14	0.004
	Controls (492)	0.41	0.5	0.09	

P-values represent the χ^2 test for genotypic association. Bold highlighted p-values remain significant (p 0.05) after correction for multiple testing using the FDR method.