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Genetic variation in *FAAH* is associated with cannabis use disorders in a young adult sample of Mexican Americans*

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Abstract

Background—Cannabis is a commonly used drug and studies have shown that a significant portion of the variation in cannabis use disorders (CUDs) is heritable. Five genes known to play a role in the endocannabinoid system and CUDs were examined in a community sample of young adult Mexican Americans (MAs): *CNR1*, *MGLL*, *FAAH*, *DAGLA,* and *DAGLB.*

Methods—Gene-based tests were run to test for association between each gene and two DSM-5 cannabis phenotypes. Subsequent linear regressions were run in PLINK using an additive model to determine which single nucleotide polymorphisms (SNPs) were driving the association.

Results—*FAAH* was significantly associated with DSM-5 cannabis use disorder group count (DSM-5 CUD) using a gene-based test ($p = 0.0035$). This association survived Bonferroni correction for multiple testing at $p < 0.004$. Post hoc analyses suggested this association was driven by two common (minor allele frequency > 5%) SNPs in moderate linkage disequilibrium, rs324420 and rs4141964, at $p = 0.0014$ and $p = 0.0023$, respectively. In both cases the minor allele increased risk for DSM-5 CUD.

Conclusions—Genetic variation in *FAAH* was associated with DSM-5 CUD in MAs. This association was primarily driven by the missense SNP rs324420. *In vitro* work has provided evidence that the risk allele generates an enzyme with decreased expression and cellular stability. Although this SNP has been previously associated with substance use in the literature, this is the first association in a young adult MA sample.

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Contributors

WEMG performed the literature review, data analysis, and drafted the manuscript. KWC contributed to the genotyping. CLE contributed to the recruitment, collection, and analysis of phenotypic and genotypic data for the sample. All authors contributed to the writing and review of this brief communication and have approved the final report.

Conflict of interest

The authors have no conflict of interest to report.

Keywords

cannabis dependence; gene-based test; human genetic association study

1. INTRODUCTION

Cannabis is the most widely used substance, second to alcohol, in the U.S. (National Institute on Drug Abuse, 2016). The brain's endogenous endocannabinoid system (ECS) is comprised of cannabinoids (endocannabinoids, eCBs), cannabinoid receptors, and enzymes responsible for the synthesis and degradation of eCBs. The two major ligands in the ECS are arachidonoyl ethanolamide (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) (see Lu and Mackie, 2016 for review). Unlike other neurotransmitters, typically synthesized and stored in synaptic vesicles, AEA and 2-AG are synthesized "on demand" from precursors present in lipid membranes. Specifically, AEA is manufactured from *N*-arachidonoyl phosphatidyl ethanol. 2-AG is produced by a two-step hydrolysis: first, an arachidonoyl-containing phosphatidyl inositol bis-phosphate is hydrolyzed into a diacylglycerol (DAG); and second, DAG is hydrolyzed into 2-AG by two DAG lipases (encoded by *DAGLA* and *DAGLB*). Once released into the synapse, eCBs primarily bind the cannabinoid type 1 and 2 receptors (CB1 and CB2, encoded by *CNR1* and *CNR2*, respectively). CB1 is the primary eCB receptor at which AEA and 2-AG have higher efficacy than at CB2 receptors. CB1 is also abundantly expressed in the central nervous system (CNS). Catabolism of AEA and 2-AG is predominantly carried out by fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGLL), respectively. Endocannabinoid receptors also bind Δ^9-tetrahydrocannabinol, the psychoactive component present in cannabis (Mechoulam and Gaoni, 1965), and can cause a myriad of changes depending on the experimental conditions. Thus, the ECS is a promising system for candidate gene (CG) studies of cannabis use disorders (CUDs).

A recent meta-analysis estimated that 51–59% of the variation in CUDs is attributed to genetic influences (Verweij et al., 2010). Linkage peaks have been observed around genes pertinent to the ECS, including *CNR1* and *MGLL* (Agrawal et al., 2008; Ehlers et al., 2010; Hopfer et al., 2007). However, CG studies on CUDs have been inconsistent (reviewed in (Agrawal and Lynskey, 2009; Buhler et al., 2015)) and genome-wide association studies on CUDs have yielded no genome-wide significant hits (Agrawal et al., 2011, 2014; Minica et al., 2015; Verweij et al., 2013), consistent with results from a recent meta-analysis (Stringer et al., 2016).

In this study, we tested the hypothesis that genetic variation in ECS-related genes is associated with CUDs in a sample of Mexican Americans (MAs). The racial/ethnic composition of the U.S. is changing rapidly with the nonwhite segment of the population expanding faster than whites. In California, the population of individuals of Hispanic heritage, who are primarily MA, is currently predicted to be the majority population by the end of the decade. Thus, understanding heath disparities in this ethnic group is a major public health concern. A recent national survey found that past year cannabis users who were Hispanic had higher odds of CUD than whites (Wu et al., 2014), yet the unique risk factors that may contribute to this risk remain largely unknown. We used a gene-based

analysis to look at genetic variation in *CNR1*, *MGLL*, *FAAH*, *DAGLA,* and *DAGLB,* based on prior evidence for association with CUDs and/or their direct role in the ECS. Specifically, two gene-based tests were used to: 1) replicate genetic associations in the literature, which have primarily focused on common variants in European Americans, and 2) investigate rare variants specific to this population of MAs for association with CUDs.

2. MATERIALS AND METHODS

Data were derived from a cohort of 619 MAs, as previously described (Ehlers et al., 2011). Briefly, these subjects were recruited using a commercial mailing list that supplied the addresses of individuals with Hispanic surnames in 11 zip codes in San Diego County. Participants were required to be of Hispanic heritage, between 18 and 30 years old, living in the U.S. legally, and able to read and write in English; exclusionary criteria included being pregnant or nursing, or having a major medical or neurological disorder or injury. Hispanic heritage was self-reported and based on the origin of each subject's 8 grandparents. 97.9% of the sample self-identified as having 12.5% or more Hispanic heritage and 91.4% as having 50% or more Hispanic heritage. 83.8% of the sample had 50% or more Mexican heritage alone.

The Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz et al., 1994), a well-documented and reliable resource for diagnosing substance use (SU) behaviors (Bucholz et al., 1994; Hesselbrock et al., 1999), was used to collect information for several cannabis phenotypes. However, given the nested nature of some phenotypes, as well as high correlations between them (e.g. the DSM-IV and DSM-5 phenotypes), we focused on the following two DSM-5 phenotypes: DSM-5 cannabis use disorder group count (DSM-5 CUD, quantitative), and having moderate or severe CUD by DSM-5 (DSM-5 MSU, dichotomous). The Institutional Review Board of the Scripps Research Institute approved the protocol for this study. Written consent was obtained from all participants.

DNA was extracted from blood samples and subsequently prepared and genotyped using the Affymetrix Exome1A chip as previously described (Norden-Krichmar et al., 2014). Initial quality control was performed according to Affymetrix best practices (Affymetrix, 2011). In addition, single nucleotide polymorphisms (SNPs) out of Hardy-Weinberg Equilibrium (HWE) at p-value$<10^{-10}$ were removed, as were SNPs with bad genotype clusters. Genome-wide Complex Trait Analysis (Yang et al., 2011) was used to remove subjects of high hidden relatedness (genetic relationship cutoff 0.125) and calculate principal components (PCs). Gender, age, and 20 PCs were included as covariates in all analyses.

Variants were annotated to genes using the Affymetrix Exome1A chip description file. No minor allele frequency (MAF) cutoff was applied. The gene test was performed in R (R Development Core Team, 2012) with the Sequence Kernel Association Test (SKAT; Wu et al., 2011) package. This test allows for variants that differ in direction and magnitude of effect. We used two specific algorithms within this method: SKAT-O, an extension of SKAT in which an optimal test is derived from a burden and typical SKAT analysis (Lee et al., 2012); and SKAT_CommonRare, in which the combined effect of rare and common variants

is tested (Ionita-Laza et al., 2014). Tests were run using all default parameters (with the exception of method="optimal.adj" as opposed to "davis" for the SKAT-O test).

Multiple test correction for each hypothesis was employed as follows. First, to correct for the number of phenotypes tested, the effective number of independent phenotypes was calculated using the variance of the eigenvalues of the phenotype correlation matrix after correction for covariates (Cheverud, 2001; Nyholt, 2004). The Bonferroni corrected significance threshold was calculated at 0.004 by dividing 0.05 by the number of genes multiplied by the effective number of independent phenotypes.

In order to determine which variants were driving the gene-wise association, post hoc analyses were performed by running linear regressions in PLINK (Purcell et al., 2007) on each SNP in the associated gene using an additive model. PLINK was used to calculate MAF and linkage disequilibrium (LD).

3. RESULTS

Five hundred and forty eight subjects (228M, 320F) were used in the analysis, 389 (70.99%) of whom had used cannabis. The mean age at the time of interview was 23.70yrs. Additional sample demographics are provided in Supplementary Table 1[1].

No genes were associated with DSM-5 CUD or DSM-5 MSU using SKAT-O (results not shown). *FAAH* was associated with DSM-5 CUD using SKAT_CommonRare (Table 1). This association survived Bonferroni correction for multiple testing at $p<0.004$. A complete list of SNPs included in each gene-based test is provided in Supplementary Table 2[2]. Lack of association using SKAT-O and association using SKAT_CommonRare suggested these results were driven by the common variants. Univariate tests with the common SNPs in *FAAH* and DSM-5 CUD confirmed this; two common SNPs, rs4141964 and rs324420, were driving the association (Table 2). These SNPs were in LD ($R^2=0.585$ and $D'=0.988$), and in both cases the minor allele was shown to increase risk for DSM-5 CUD.

4. DISCUSSION

In the present study, five genes known to play a role in the ECS were tested for association with CUDs using a gene-based test; *FAAH* was associated with DSM-5 CUD after correction for multiple testing. Two previous studies have examined genetic variation in targeted ECS genes using a gene-based test; however, *FAAH* was not associated with cannabis use (Verweij et al., 2012) or cannabis dependence (Carey et al., 2015). This finding is not altogether surprising, given that these phenotypes differ from the construct used in the present study, and our MA subjects represent an ethnically unique sample at high risk for developing alcoholism (Criado and Ehlers, 2007; Ehlers and Phillips, 2007). Indeed most subjects with DSM-5 MSU had a comorbid DSM-5 alcohol use disorder.

[1]Supplementary material can be found by accessing the online version of this paper at http://dx.doi.org and by entering doi:...
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Subsequent analyses revealed two correlated SNPs driving the association with *FAAH* and DSM-5 CUD: rs324420, a missense SNP resulting in the conversion of a proline to a threonine; and rs4141964, an intronic SNP. Although some studies have not detected an association between rs324420 and SU (Haughey et al., 2008; Proudnikov et al., 2010; Verweij et al., 2012), particularly in Asian populations (Iwasaki et al., 2007; Morita et al., 2005), the literature overwhelmingly supports a role for rs324420 in SU (Buhler et al., 2014; Flanagan et al., 2006). While one study found that subjects homozygous for the minor allele were 0.25 times less likely to be cannabis dependent (Tyndale et al., 2007), our results suggesting the A allele increases risk for CUDs are in line with previous findings (Li et al., 2011; Sipe et al., 2002). In addition, the A allele produces a defective mutant enzyme with reduced expression and stability (Chiang et al., 2004). Only one study investigated the effect of rs4141964 in SU; in a multi-ethnic study, Bidwell and colleagues (2013) found a significant main effect of a *FAAH* haplotype containing the minor alleles of rs4141964 and rs324420 that predicted higher marijuana-related problems. Thus, our results concur with the current literature and suggest that the minor alleles of rs4141964 and rs324420 are associated with CUDs.

CNR2 was not included in the original analysis because it is primarily expressed in the periphery (Munro et al., 1993). However, a post hoc gene-test was run on *CNR2* based on accumulating evidence for *CNR2* expression in the CNS (reviewed in (Onaivi et al., 2006a)) and involvement in SU (Ishiguro et al., 2007; Onaivi et al., 2006b, 2008). Although only three SNPs were included, *CNR2* was not associated with DSM-5 CUD or MSU (Supplementary Table 3[3]).

This study has several strengths and limitations. Gene-based tests are a more powerful alternative to single SNP tests, and by testing specific CGs we sidestepped several drawbacks when examining already curated gene tests (Wang et al., 2011). SKAT has higher power than several other burden tests to detect genetic effects (Wu et al., 2011). However, we were limited by what SNPs were genotyped on the Affymetrix Exome1A chip and were annotated to our target genes by the Affymetrix Exome1A chip description file, and thus important signals may have been missed in each gene. Due to the modest sample size, which could lead to false negatives, subjects who had never used cannabis were included. There are two reasons why this likely does not mitigate the results of this study: 1) a recent meta-analysis suggested that environmental influences play a larger role in cannabis initiation than CUD (Verweij et al., 2010), and 2) there may be some overlap in genetic influences between cannabis initiation and CUD (Agrawal et al., 2005; Fowler et al., 2007; Gillespie et al., 2009). Finally, as with other human genetic studies, replication is needed to support our findings. However, the exclusivity of the sample is both a strength and limitation; this sample is primarily of MA ancestry and has been previously shown to have a high risk of developing alcoholism (Criado and Ehlers, 2007; Ehlers and Phillips, 2007).

The present study provided evidence for a role of *FAAH* in CUDs in MAs. This association was primarily driven by a missense SNP in *FAAH* that has been previously associated with

[3]Supplementary material can be found by accessing the online version of this paper at http://dx.doi.org and by entering doi:...

SU in the literature and shown to influence enzyme stability. This is the first association between SNPs in *FAAH* and DSM-5 CUD in a young adult MA sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Genetic variation in *FAAH* was associated with DSM-5 cannabis use disorder.
- *CNR1, MGLL, DAGLA*, and *DAGLB* were not associated with DSM-5 cannabis use disorder.
- Previous associations with rs324420 and cannabis use disorders were replicated.

Table 1

SKAT_CommonRare results.

		DSM-5 CUD		DSM-5 MSU	
Gene	**# Tested markers (%rare[a])**	**Q[b]**	**P-value**	**Q**	**P-value**
CNR1	5 (0%)	306.68	0.2736	34.571	0.3241
FAAH	6 (66.67%)	5.728	0.0035[c]	2.499	0.1066
MGLL	6 (83.33%)	0.989	0.6195	1.051	0.5675
DAGLA	4 (75%)	2.535	0.0898	2.666	0.0814
DAGLB	9 (77.78%)	1.236	0.6352	1.167	0.6526

[a]The MAF cutoff for common vs rare variants is calculated in SKAT as follows: 1/ √[2 SampleSize]

[b]The test statistic of SKAT

[c]Survived Bonferroni correction for multiple testing at p < 0.004

Table 2

FAAH SNP results with DSM-5 CUD.

SNP	Alleles[a]	Beta[b]	STAT	P	MAF (sample)	MAF (1000G AMR)[c]
rs4141964	C/T	0.4534	3.062	0.0023	0.4413	49%
rs324420	C/A	0.5115	3.203	0.0014	0.3203	35%

[a] Major/minor

[b] A positive regression coefficient means that the minor allele is interpreted as the risk allele

[c] 1000 Genomes Project Phase 3 MAF for the American population retrieved from the 1000 Genomes Browser (http://browser.1000genomes.org/index.html)