ORIGINAL ARTICLE

An Autosomal Linkage Scan for Cannabis Use Disorders in the Nicotine Addiction Genetics Project

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Context: Despite accumulating evidence that there is a genetic basis for cannabis use disorders (ie, abuse and dependence), few studies have identified genomic regions that may harbor biological risk and protective factors.

Objective: To conduct autosomal linkage analyses that identify genomic regions that may harbor genes conferring a vulnerability to cannabis use disorders.

Design: In 289 Australian families who participated in the Nicotine Addiction Genetics Project, 423 autosomal markers were genotyped. Families were ascertained for heavy cigarette smoking. Linkage was conducted for *DSM-IV* cannabis dependence and for a novel factor score representing problems with cannabis use, including occurrence of 3 of 4 abuse criteria (excluding legal problems) and 6 *DSM-IV* dependence criteria.

Results: A maximum logarithm of odds (LOD) of 3.36 was noted for the cannabis problems factor score on chromosome arm 1p. An LOD of 2.2 was noted on chromosome 4 in the region of the γ-aminobutyric acid type A gene cluster, including *GABRA2*, which has been implicated in drug use disorders. For *DSM-IV* cannabis dependence, a modest LOD score on chromosome 6 (1.42) near cannabinoid receptor 1 (*CNR1*) was identified. In addition, support for an elevation on chromosome 3, identified in prior independent studies, was noted for the factor score and cannabis dependence (LOD, 1.4).

Conclusions: Genes such as *ELTD1* on chromosome 1, in addition to genes on chromosomes 4 (eg, *GABRA2*) and 6 (eg, *CNR1*), may be associated with the genetic risk for cannabis use disorders. We introduce a novel quantitative phenotype, a cannabis problems factor score composed of *DSM-IV* abuse and dependence criteria, that may be useful for future linkage and association studies.

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Cannabis is the most commonly used illicit drug, with nearly 40% of those who reported lifetime cannabis use also meeting criteria for a lifetime history of *DSM-IV* cannabis abuse and/or dependence.[1-3] It has also been reported that, although the prevalence of cannabis use in the United States has remained steady during the past decade, there has been an 18% increase in rates of cannabis use disorders among users.[4] In 2000, there were 236 400 admissions to US public treatment services for which the primary drug problem was identified as cannabis use.[5] Both experiments[6] and surveys[7] have found evidence of cognitive and psychomotor impairments secondary to cannabis use. The public health implications of long-term cannabis use are becoming increasingly clear. For instance, recent population-based surveys report that motor vehicle crash injuries were significantly associated with habitual cannabis use, even after controlling for other risk factors (eg, blood alcohol levels).[8,9] Moreover, frequent cannabis smoking is associated with incremental impairment in lung functioning.[10] Finally, individuals with cannabis use disorders are significantly more likely to also report higher rates of psychiatric and other substance-related problems,[11-15] particularly, nicotine dependence.[16,17] Gaining a greater understanding of the genetic and environmental factors associated with cannabis-related disorders may shed light on this growing problem.

Both the twin method and, more recently, genomic approaches have helped uncover the role of genetic influence on susceptibility to cannabis-related problems. Family and twin studies highlight the important role of heritable influences on the development of cannabis use disorders. In twin studies, 50% to 75% of the total variance in cannabis abuse/dependence has been attributed to genetic influences.[18] Despite the moderate to high heritability of cannabis use disorders, only 2 linkage studies have attempted to identify genomic regions associated with cannabis use disorders. In their

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study of adolescents and young adults aged 12 to 25 years recruited from treatment centers and the community, Hopfer et al[19] found evidence for linkage on chromosomes 3 and 9 for a quantitative cannabis dependence symptom count (logarithm of odds [LOD], 2.61 and 2.57). Not surprisingly, these linkage peaks coincided with linkage peaks for antisocial behavior and drug dependence identified previously in the same sample.[20,21] A recent report that used data on adult alcoholics and their family members from the Collaborative Study on the Genetics of Alcoholism (COGA) identified a linkage peak on chromosome 14 (LOD, 1.92) for cannabis dependence symptoms.[22]

The *DSM-IV* defines cannabis dependence as occurrence of 3 of 6 dependence symptoms, excluding withdrawal, that cluster within a single 12-month period.[23] Furthermore, the *DSM-IV* imposes a hierarchy on dependence, such that individuals qualifying for a diagnosis of dependence are not eligible for a diagnosis of *DSM-IV* abuse (ie, occurrence of 1 of 4 abuse symptoms). Although this is the clinically accepted definition of cannabis dependence, recent psychometric studies have demonstrated that symptoms of both cannabis abuse and dependence are indexed by a single underlying dimension of liability.[3,24-26] This underlying abuse/dependence factor is a dimensional view of risk to cannabis abuse/dependence and is of particular utility as a linkage phenotype because it includes all cannabis-exposed individuals, not only the affected sibling pairs, in pedigrees with phenotypic data.

The aim of the present study was to conduct linkage analyses on data from 289 families of adult Australians ascertained using a heavy tobacco use phenotype as part of the Nicotine Addiction Genetics Project (hereinafter called NAG).[27] The other arm of the NAG includes families of adult Finnish smokers, ascertained with the same scheme. Because cannabis rarely was used by this Finnish cohort (<5% lifetime use), data from this arm of the project were not included in these analyses. In addition to a diagnostic assessment of cannabis dependence (affected vs unaffected), we also conducted linkage analyses on a novel quantitative factor score as a continuous measure of vulnerability to cannabis use problems.

METHODS

The current data are part of the NAG, a large multisite family-based genetic study of tobacco use and related behaviors. Extensive details regarding the ascertainment procedure, characteristics of the NAG samples used for linkage analysis and of a community-based sample used to generate scoring coefficients, and the genotyping procedure were previously reported by Saccone et al.[27] All data collection procedures were approved by the institutional review boards at Washington University, the Queensland Institute of Medical Research, and the University of Helsinki. Participants gave informed consent for an interview, for providing a blood sample for DNA extraction and cell lines, and for sharing their anonymous clinical and genotypic records and DNA with scientists other than the project's investigators. The data collection protocol included telephone screening and a diagnostic interview, after which a sample of blood was requested for DNA extraction. Eligible families from both sites were required to have at least 1 adult sibling pair (not including monozygotic twin pairs) concordant for a history of cigarette smoking, which was determined by earlier questionnaire or interview surveys.

AUSTRALIAN NAG

Families of primarily Anglo-Celtic or Northern European ancestry (>90% of participants) were identified from 2 cohorts of same-sex male and female and opposite-sex adult twins and a sample of spouses of the older of 2 cohorts of twins associated with the Australian National Health and Medical Research Council.[28,29] Families were targeted for screening if information from previous surveys suggested that they included at least 1 sibling pair concordant for a lifetime history of heavy smoking (defined as either a history of smoking 20 cigarettes per day during the period of heaviest use or smoking at least 40 cigarettes in any 24-hour period). Families with available biological parents were prioritized, and in the absence of data from both parents, an attempt was made to obtain clinical data and a blood sample from at least 1 unaffected full biological sibling. Sample age range was 21 to 86 years. For linkage analyses, families were prioritized for genotyping based on provision of DNA by 1 or both parents and the number of additional full siblings with DNA who reported a lifetime history of heavy cigarette smoking (in addition to the required criterion of 2 siblings who were heavy smokers). Blood samples for genotyping from the index case and all consenting family members were drawn at local health center laboratories, outpatient clinics, and by primary care physicians. The DNA was extracted, stored, and genotyped at the Australian Genome Research Facility.

During the screening interview, sufficient information on lifetime history of cigarette smoking was obtained from the index case to confirm family eligibility, and permission was requested to contact available parents and full biological siblings for participation in the study.

FINNISH NAG

Although they are not included in the present analyses, the NAG also includes a Finnish component. Because of the high mean age of the index twins, the number of included parents was quite small, and they were not asked about their illicit drug use.

At both sites, during the screening interview, sufficient information on lifetime history of cigarette smoking was obtained from the index case to confirm family eligibility, and permission was requested to contact available parents and full biological siblings for participation in the study.

BigSib

Because of oversampling for nicotine-related phenotypes in the NAG, we used data from a general community-based sample (BigSib) to generate phenotypic scoring coefficients for the Australian NAG, thus allowing for generalization of our linkage findings to the general population. The BigSib (age range, 18-91 years), so called because it consists of 1254 families of predominantly Anglo-Celtic or Northern European ancestry, each with 5 or more offspring with the same biological parents, is a community sample that was ascertained for sibship size, not alcohol- or tobacco-related behaviors, other substance-related problems, or psychopathological characteristics. As discussed in some detail by Saccone et al,[27] the BigSib was also drawn from the Australian Twin Registry and assessed using an interview protocol that was largely similar to the NAG, and was identical for the cigarette smoking and cannabis use behaviors sections. The BigSib was used to score the phenotypic data; the current analyses do not use genotypic data from BigSib participants who do not overlap with the NAG.

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Table 1. Cannabis Use and Symptoms of *DSM-IV* Abuse and Dependence Among Lifetime Users[a]

Characteristic	NAG (n=948)[b]		BigSib (n=1254)	
	M	F	M	F
No. of subjects, including parents	1629	1802	2572	3204
No. of subjects with phenotypic data, excluding parents	1234	1270	2426	3028
Lifetime cannabis use	70.6	66.5	56.3	42.9
Used ≥11 times	42.0	29.8	27.9	16.0
Median age at first use, y	18.0	18.0	18.0	19.0
DSM-IV Abuse	26.1	14.1	19.9	10.2
DSM-IV Abuse criteria[c]				
A1: Failure to fulfill major role obligations	10.2	5.2	8.6	4.5
A2: Use in hazardous situations	21.1	10.3	17.9	7.5
A3: Legal problems	1.8	0.4	0.7[d]	0.2[d]
A4: Continued use despite recurrent social/interpersonal problems[e]	7.9	5.2	5.7[d]	4.2[d]
DSM-IV Dependence	14.3	9.4	10	6.5
DSM-IV Dependence criteria[c,f]				
D1: Tolerance	24.0	15.1	17.2	10.1
D3: Taken in larger amounts/longer than intended	17.7	12.8	13.5	8.4
D4: Repeated unsuccessful attempts to quit/cut back[g]	17.2	13.4	13.2	9.2
D5: Time spent obtaining/using cannabis	9.2	5.3	6.8	3.4
D6: Give up important activities to use	9.2	6.0	6.4	4.0
D7: Persistent use despite physical/psychological problems[h]	14.5	8.8	10.9	6.5
No. of abuse/dependence criteria, mean	1.3	0.8	1.0	0.6

Abbreviations: BigSib, a general community-based sample; F, female; M, male; NAG, Nicotine Addiction Genetics Project.
[a] Data are given as the percentage of participants, unless otherwise indicated.
[b] The NAG includes 289 families (590 men and 721 women) for whom genotypic and clinical data were available.
[c] *DSM-IV* criteria were constructed based on dichotomous responses to 13 items. Unless otherwise noted, a single item assessed the criterion.
[d] Not significantly different in men vs women; all other estimates in men were higher than estimates in women ($P < .05$).
[e] Assessed by 2 items: 3 or more separate occasions when use caused problems with family or friends; 3 or more separate occasions when got into physical fight while under the influence of cannabis
[f] D2 (withdrawal) is not a *DSM-IV* dependence symptom for cannabis.
[g] Assessed by 2 items: 3 or more times wanted to stop or cut back; 2 or more times tried to stop or cut down and was unable.
[h] Assessed by 2 items: continue to use despite health problems; continue to use despite emotional or psychological problems.

PHENOTYPES

The NAG and BigSib participants reported occurrence of *DSM-IV* symptoms of cannabis abuse and dependence.[23] As part of an extensive computer-assisted telephone interview, diagnostic assessments on cannabis-related behaviors were assessed using an adapted version of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA)[30,31] for telephone administration. The tobacco section of the computer-assisted telephone interview was derived from the Composite International Diagnostic Interview (CIDI).[32] Participants in NAG and BigSib were asked 4 abuse and 6 dependence questions based on *DSM-IV* criteria. Only those who reported using cannabis 11 or more times were asked to answer questions about abuse and dependence criteria. Therefore, for the purposes of the present analyses, individuals who reported a lifetime history of cannabis use but had used cannabis fewer than 11 times were coded 0 for individual *DSM-IV* criteria, whereas those who had never used cannabis were coded as missing. In the present study, linkage analyses were conducted on 2 measures of cannabis dependence:

1. A diagnostic measure of *DSM-IV* cannabis dependence, for which an individual met lifetime criteria if he or she had endorsed 3 (or more) of 6 *DSM-IV* dependence symptoms and if these symptoms clustered within a single 12-month period. The phenotype was coded dichotomously, such that all individuals meeting criteria for *DSM-IV* cannabis dependence were coded as affected, all individuals reporting a lifetime history of cannabis use but not meeting criteria for dependence (endorsing 0, 1, or 2 criteria) were coded as unaffected, whereas those who had never used cannabis even once in their lifetime were coded as missing.

2. We also conducted a factor analysis of *DSM-IV* abuse and dependence criteria for those reporting a lifetime history of cannabis use. Criteria, which are listed in **Table 1**, were constructed using dichotomous responses (0/1) to 13 items (see the footnotes to Table 1 for item-to-criterion correspondence). The factor score resulting from this analysis was used as the quantitative trait for linkage analyses. In general, normally distributed quantitative phenotypes are more informative than their dichotomous counterparts for genomic analyses because they do not rely on thresholds (for instance, affection status on the endorsement of at least 3 dependence symptoms) that are often of questionable psychometric validity[3,24-26] and, unlike an affected sib-pair analysis, which only uses data from families with affected offspring, quantitative traits include data from all individuals across all available families (in our study, anyone exposed to cannabis use). Unlike in the COGA study, a simple symptom count was not used for the present analyses.

GENOTYPING

An ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, California) was used to type the ABI PRISM, version 11, marker set of 381 autosomal microsatellite markers spaced approximately 10 cM apart along the genome. Mean marker heterozygosity was 0.79. Checks for mendelian errors were performed using RELCHECK[33,34] and PREST,[35] and inconsistent geno-

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types were rectified or excluded from analyses. Nonmendelian errors included the identification of 3 sets of monozygotic twins (deleted) and the identification of a case of nonpaternity. We analyzed these markers separately, to account for the hemizygous nature of X-chromosome markers in men. The marker call rate was predominantly more than 80%, and the average error rate was 0.0045. To provide additional information for linkage to nicotine-related phenotypes, 2 additional microsatellites on chromosome 22 and 40 single-nucleotide polymorphisms (SNPs) were added to the marker set (27 on chromosome 5 and 13 on chromosome 22). We included these microsatellites and SNPs, which were genotyped at Washington University, in our analyses. Chromosome 5 SNPs were typed with the SNPlex Genotyping System (Applied Biosystems). We used a Web-based assay design (Applied Biosystems) to generate SNP-specific ligation probes. The SNP-specific ligation probes were then phosphorylated and bound to allele-specific oligo linkers. All oligo-ligation products were purified with an exonuclease reaction to eliminate unligated probes, and then underwent polymerase chain reaction amplification with universal biotinylated primers. The biotinylated amplicons were bound to streptavidin-coated plates and then hybridized with fluorescence-labeled ZipChute probes (SNPlex Genotyping System). The ZipChute probes attached to biotinylated amplicons were eluted and electrophoresed using an ABI 3730 sequencer (Applied Biosystems). Genotype spectra were analyzed with GeneMapper software, version 4.0 (Applied Biosystems). Chromosome 22 SNPs were typed with the MassARRAY system (Sequenom, San Diego, California). This protocol is described in detail elsewhere.[36]

FACTOR ANALYSIS

To construct the cannabis use disorders factor score, we conducted principal factor analyses, separately by sex (as prior analyses have suggested the possibility of sex heterogeneity in these items[3]), using SAS statistical software (SAS Institute Inc, Cary, North Carolina).[37] Factor loadings for the 4 abuse and 6 dependence criteria were compared for both NAG and BigSib, along with other fit statistics, to construct the cannabis use disorders factor score in the NAG. However, because these scores were from a sample ascertained for tobacco smoking, we used a scoring procedure to adjust for ascertainment effects. The continuous factor score (not a sum score) representing the unidimensional latent construct underlying the abuse/dependence items was used for analyses.

REGRESSION AND SCORING COEFFICIENTS

First, we tested for the linear and quadratic effects of age. Then, we regressed out the significant linear effects of age (β, –0.02; SE, 0.05) from the factor score computed in the BigSib. Because the factors were constructed separately in men and women, sex was not included as a covariate in the regression equation. The regression coefficients from BigSib were then applied to the factor score computed for NAG. The resulting residualized factor score for cannabis use disorders among NAG participants, with the effects of age regressed out, were standardized with respect to the distribution of the factor score from BigSib. To obtain the cannabis use disorders factor score used for the linkage analyses (mean, 0; SD, 1.0; which aids with model specification for linkage), the resulting scored NAG data were divided by the standard deviation of the BigSib residual.

LINKAGE ANALYSIS

Linkage analyses were conducted on 289 families of European ancestry. Using the 383 microsatellites typed for linkage, there was no evidence for population stratification (using STRUCTURE[38]) within this sample. The total genotyped sample consisted of 1341 individuals, including 394 parents. Of these genotyped individuals, 709 siblings (from 793 possible pairings of siblings across 289 families) were informative for the quantitative trait, and 19 families, including 25 affected sibling pairs, were informative for *DSM-IV* cannabis dependence. Because parents were not asked about their illicit drug use, parental phenotypic data were unavailable. Mean family size, including parents, was 4.7 people. All linkage analyses were conducted using the genetic software package MERLIN,[39] which uses gene flow tree-based pedigree analysis for rapid and efficient linkage analysis. A centimorgan marker map, using deCODE positions for microsatellite markers (deCODE genetics, Reykjavik, Iceland), was used (with extrapolation to centimorgan position for 50 fine-mapping SNPs, using National Center for Biotechnology Information build 36.2 physical positions). The nonparametric linkage option in MERLIN[39] was used for nonparametric single- and multipoint linkage analysis of the dichotomous *DSM-IV* cannabis dependence diagnosis.

We used MERLIN-REGRESS, which allows for user-specified means, variances, and estimates of heritability, to conduct single- and multipoint linkage analysis of the quantitative factor score of cannabis abuse and dependence criteria. This method involves a regression of the extent of identity by descent sharing between family members on the squared sums and squared differences of trait scores. Simulation studies have shown this technique to be fairly robust to type I error rate, sibship size (when marker informativeness is high), and nonnormality, as well as misspecifications of the mean, variance, and heritability of the trait that need to be specified for the analyses.[40] Misspecifications of mean and variance influence power to detect linkage, although they do not inflate type I error rates. Furthermore, by generating scoring coefficients in the community-based BigSib sample and applying them to the Australian component of the NAG, we were able to specify means and variances that were applicable to the general population; therefore, our LOD scores are not specific to a sample in which families were ascertained for heavy smoking. The mean of the residuals was 0, with a variance of 1.0. Heritability was specified at 0.5, based on published estimates of heritable influences on cannabis dependence among Australian adults.[41]

GENOME-WIDE SIGNIFICANCE LEVEL

To compute the probability of observing a LOD score of a given magnitude by chance alone, we simulated 1000 data sets using the gene-dropping algorithm in MERLIN. This involves retaining the phenotypic assignments and randomly shuffling genotypes while maintaining marker frequencies and segregation patterns with families. Linkage analyses were conducted on each of the simulated data sets. The genome-wide significance level, represented by the *P* value, was computed using the conservative $P = r/1001$, where r is the number of times a LOD score greater than or equal to the observed maximum LOD is noted in a simulated sample.[42]

RESULTS

SAMPLE CHARACTERISTICS

Table 1 shows the prevalence of cannabis use and of individual symptoms of cannabis abuse and dependence in siblings from NAG and BigSib by sex. Lifetime cannabis use was reported by 70.6% and 66.5% of men and women in the Australian NAG and by 56.3% of men and

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Table 2. Standardized Factor Loadings for *DSM-IV* Cannabis Abuse and Dependence

DSM-IV Criteria	NAG		BigSib	
	M	F	M	F
Abuse				
A1: Failure to fulfill major role obligations	0.68	0.55	0.66	0.57
A2: Use in hazardous situations	0.57	0.59	0.61	0.59
A3: Legal problems	0.17	0.14	0.17	0.07
A4: Continued use despite recurrent social/interpersonal problems	0.62	0.57	0.64	0.62
Dependence[a]				
D1: Tolerance	0.65	0.73	0.70	0.67
D3: Taken in larger amounts/longer than intended	0.65	0.70	0.67	0.73
D4: Repeated unsuccessful attempts to quit/cut back	0.66	0.68	0.69	0.71
D5: Time spent obtaining/using cannabis	0.66	0.68	0.72	0.64
D6: Give up important activities to use	0.69	0.69	0.70	0.66
D7: Persistent use despite physical/psychological problems	0.65	0.65	0.62	0.64

Abbreviations: BigSib, a general community-based sample; F, female; M, male; NAG, Nicotine Addiction Genetics Project.
[a] D2 (withdrawal) is not a *DSM-IV* dependence symptom for cannabis.

42.9% of women in BigSib. The prevalence of cannabis use in BigSib was comparable to national norms (about 42%-64% in individuals aged 20-50 years)[43] although the prevalence in NAG was somewhat higher. Of NAG participants who reported lifetime cannabis use, 59.5% of men and 44.9% of women (49.7% and 37.3%, respectively, in BigSib) also reported using cannabis at least 11 times. (See Table 1 for prevalence in all individuals.) Men reported first using cannabis at a somewhat earlier age than women (Table 1). The most common abuse/dependence symptom among lifetime users was tolerance (10.1%-24.0%), whereas legal problems were the least common symptom (0.2%-1.8%). More men than women met criteria for a lifetime history of *DSM-IV* cannabis dependence, the prevalence of which was 14.3% and 9.4% among NAG male and female lifetime users, respectively. For BigSib, cannabis dependence was diagnosed more often among men (10%) than women (6.5%).

Even before they first used cannabis, 86.7% of NAG participants and 73.4% of BigSib participants reported having the opportunity to use cannabis (even if they did not use it at that time). Men (BigSib, 81.9%; NAG, 88.7%) were more likely than women (BigSib, 68.2%; NAG, 85.0%) to report having that opportunity. Participants in BigSib reported a somewhat higher mean age at first exposure (19.5 years for men and 20.3 for women) than did the NAG participants (16.5 years for men and women).

In contrast, only 5% of men and 3% of women from the Finnish NAG sample (n=88) reported any lifetime use of cannabis; among them only 9 men (12%) and 2 women (7%) had used cannabis 11 times or more. Because there were so few such cannabis users (0.4% of all participants), no further genetic analyses were undertaken for this group.

FACTOR ANALYSIS OF ABUSE AND DEPENDENCE CRITERIA

In both the Australian NAG and BigSib, the abuse and dependence criteria were indexed by a single underlying factor, which explained from 68% to 75% of the total variance (eigenvalues ≥6.4). The Tucker and Lewis Reliability Coefficient was 0.90 or more across samples (NAG and BigSib) and sex. Visual inspection of scree plots also confirmed the choice of a single-factor solution. With the exception of the legal criterion, abuse and dependence criteria demonstrated high and comparable factor loadings, ranging from 0.55 to 0.73. The factor structure was similar among men and women (**Table 2**), with no evidence for sex differences in item loadings. The legal criterion, as assessed by being arrested for a cannabis-related offense on 2 or more occasions, did not load well on the underlying factor in either sample or sex, and consequently was dropped from the factor analysis (loadings, 0.07-0.17) (Table 2) . Hence, the cannabis problems factor score was a composite of 3 abuse (hazard, failure, and continue) and 6 dependence criteria.

LINKAGE ANALYSES

Cannabis Problems Factor

The strongest linkage signal was found on chromosome 1 with this quantitative abuse/dependence factor score. A LOD score of 3.36 (102 cM) was noted in multipoint regression analyses. The comparable maximum LOD from the single-point analysis was 2.0 at microsatellite marker D1S2841, situated near the epidermal growth factor, latrophilin, and 7 transmembrane domain containing 1 gene (*ELTD1*) (**Figure 1**). An additional finding potentially of biological relevance was noted on chromosome 4 (LOD, 2.22 at 38 cM). This region harbors a cluster of 4 *GABRA* genes, including *GABRA2*, which has been associated with dependence on alcohol, cannabis, and other illicit drugs (**Figure 2**).

DSM-IV Cannabis Dependence

Using the stringent threshold of more than 3 criteria that cluster in a single 12-month period to ascribe *DSM-IV* cannabis dependence, only 19 genotyped families with

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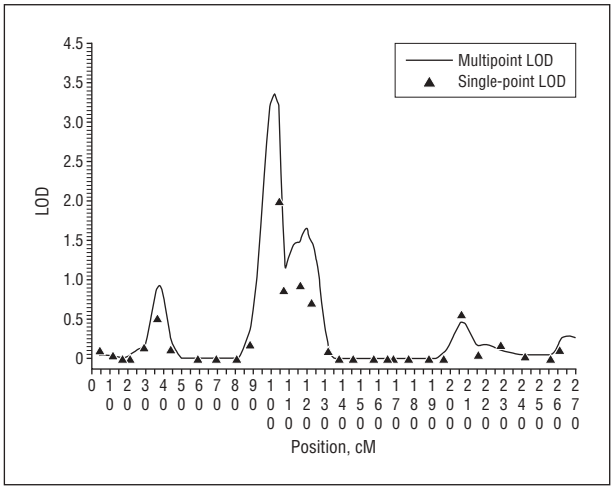

Figure 1. Linkage identifies a novel region associated with cannabis abuse/dependence on chromosome arm 1p in a sample of Australian adults ascertained for heavy tobacco use. LOD indicates logarithm of odds.

affected sibling pairs were identified for nonparametric linkage. **Table 3** shows multipoint LOD scores that exceeded 1.3 with this dichotomous definition. Of significant note is the maximum LOD of 2.23 on the end of the q arm of chromosome 17 (approximately 134.6 cM). Other regions of potential interest included chromosome 3 (LOD, 1.41 at 154.9 cM) and chromosome 6 (LOD, 1.42 at 105.2 cM).

Genome-Wide Significance

After conducting linkage analyses on 1000 simulated data sets, we found 100 instances in which the LOD score exceeded a threshold of 3.4. Hence, our maximum LOD of 3.36 is significant at $P \approx .10$.

COMMENT

We sought to identify genomic regions that may harbor genes conferring vulnerability to cannabis dependence in a sample of adult Australian men and women. A LOD score of 1.3 was obtained in a region noted by a previous study (on chromosome 3). Linkage to a region of known gene association (on chromosome 4) was found. In addition to findings consistent with previously identified regions for drug dependence, there was support for a novel region on chromosome 1 with a LOD score of 3.36 for a quantitative measure of cannabis abuse/dependence.

The LOD score of 3.36 on chromosome arm 1p identifies a novel genomic region for cannabis use problems. There was evidence for linkage (LOD, 2.9) even when those who had not used cannabis 11 or more times were excluded from the analyses. The microsatellite marker D1S2841, which contributes to the maximum single-point LOD score in this location, lies in the *ELTD1* (EGF, latrophilin, and 7 transmembrane domain containing 1) gene. Relatively little is known of this 151-kb gene, which belongs to the secretin family of G-protein coupled receptors and encodes G-protein coupled receptors and EGF-finding calcium domains.[44] *ELTD1* has been implicated in neuropeptide signaling and signal transduction pathways,[45] making it an important candidate. Cross-species conservation in the exonic sequence also underscores its possible importance.

The phenotype used by us for the quantitative linkage analyses warrants discussion. Consistent with an accumulating body of evidence,[3,24-26,46,47] we found considerable support for a unidimensional continuum of risk underlying *DSM-IV* criteria of both abuse and dependence (cannabis problems factor). Most notably, our results are fairly similar to those in a previous study of Australian adults by Teesson et al[26]; they also found evidence for the lowest factor loading for the legal criterion. In our analyses, however, we dropped the legal criterion because it was the only criterion with poor factor loading on the underlying factor. This factor pattern was replicated even when those who did not report using cannabis 11 or more times in their lifetime were excluded from the analyses. In his review of cannabis abuse/dependence criteria, Budney[48] also reports that across several studies the legal criterion were found to have limited reliability and validity.

The 1-LOD support for the region on chromosome 1 extends from 96 to 106 cM and falls within a region previously identified for alcoholism risk in the COGA. Reich et al[49] report increased allele sharing at D1S1588, which is approximately 10 cM distal from our region of 1-LOD support. Subsequently, elevated LOD scores in this region have been reported in the COGA replication sample for alcoholism, for alcoholism combined with affective disorders, and for self-rating of effects of alcohol the first 5 times it was consumed. Reich et al[49] also fine-mapped a region extending from 101.48 to 130.73 cM (approximately 94-120 cM on the deCODE map used in this study) and found association with markers at approximately 126 cM on the Marshfield map (about 116 cM on the deCODE map) and alcohol dependence. However, no previous study has found evidence for linkage in this region for illicit drug dependence. The degree to which this genetic variation is specific to cannabis use disorders or may represent the genetic liability to general substance-related problems requires further attention.

Two other studies have described linkage peaks for cannabis dependence. Hopfer et al[19] identified a region on chromosome arm 3q (spanning 131-142 cM) with a maximum LOD of 2.6 for a count of cannabis dependence symptoms in a sample of adolescents and young adults aged 12 to 25 years. Agrawal et al[22] used a similar sum score of dependence criteria from the COGA to identify a LOD of 1.92 on chromosome 14 (92 cM). The present study provided additional support for the linkage finding on chromosome arm 3q with the dichotomous cannabis dependence phenotype but not the finding from COGA on chromosome 14. We also did not replicate the linkage peak identified on chromosome arm 9q by Hopfer et al.[19]

Evidence for linkage in regions previously identified in a sample ascertained for externalizing disorders[20] is encouraging because our sample was ascertained for heavy smoking. We did not, however, replicate LOD scores from COGA. Possible reasons for this include variations in phenotype definition (symptom count in COGA) and that

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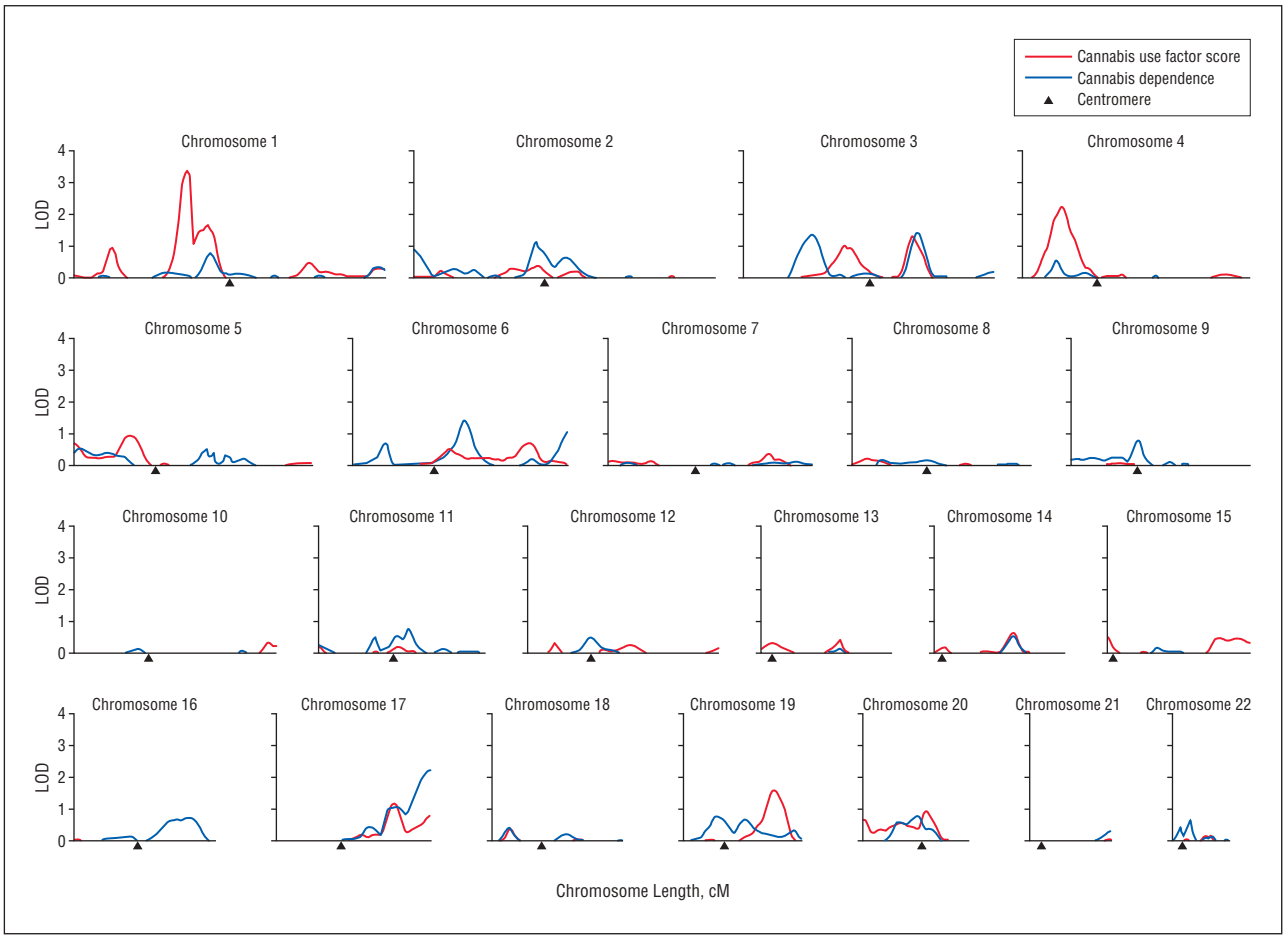

Figure 2. Multipoint linkage plots for the autosomal chromosomes for *DSM-IV* cannabis dependence (blue) and for the cannabis use disorder factor score (red). The x-axis shows length in centimorgans (plots are sized relative to the actual length of the chromosome; ie, chromosome 22 has the shortest x-axis). The y-axis shows logarithm of odds (LOD) scores from 0 to 4 with the solid lines representing LOD scores of 1, 2, and 3.

COGA, as a consequence of oversampling for alcoholism (41% of participants in COGA vs 31% in the NAG linkage sample), also oversampled for cannabis (20% of participants in COGA vs 9.5% in the NAG linkage sample) and other illicit drug dependence. The relatively lower rates of substance dependence, excluding nicotine, in our sample compared with the Colorado sample,[20] in which cannabis dependence was reported by 61% and 18% of the probands and their siblings, respectively, may have contributed to our lack of convergent findings on chromosome 9. Rates of other illicit drug use in NAG (cocaine, 0.5%; stimulants, 3.7%; sedatives, 1%; and opiates, 2.6%) were also lower than those reported by COGA (cocaine, 14%; stimulants, 9.9%; sedatives, 5.7%; and opiates, 4.6%).[22,50] However, compared with the community-based BigSib, rates of *DSM-IV* dependence were higher in the Australian arm of the NAG (nicotine, 34.9% vs 75%; alcohol, 18.4% vs 31%; and cannabis, 4% vs 9.5%). Despite these differences in sample characteristics between our study and previous studies of cannabis use behaviors, it is promising that we found support for the linkage peak on chromosome 3.

A LOD of 2.22 at 38.4 cM was noted on chromosome 4. This region on 4p harbors the γ-aminobutyric acid type A cluster, consisting of *GABRA2*, *GABRA4*, *GABRB1*, and *GABRG1*. Numerous studies[50-54] have found evidence for an association between SNPs in *GABRA2* and risk of developing alcoholism. Two of these studies report an association between these SNPs and susceptibility to drug dependence. Specifically, 1 previous study[50] has demonstrated an association between *GABRA2* and *DSM-IV* can-

Table 3. Multipoint Linkage Results for Cannabis Use Disorders in the NAG Australian Sample

Chromosome	Position, cM	Nearest Marker	LOD Score
Cannabis problems factor			
1	102.0	D1S2841[a]	3.36
3	150.9	D3S1569	1.30
4	38.4	D4S419	2.22
19	87.4	D19S571	1.60
DSM-IV cannabis dependence diagnosis			
3	154.9	D3S1569	1.41
6	105.2	D6S422	1.42
17	134.6	D17S928	2.23

Abbreviations: LOD, logarithm of odds; NAG, Nicotine Addiction Genetics Project.
[a] Single-point linkage at D1S2841 is 1.99, flanking markers D1S230 (LOD, 0.18) and D1S207 (LOD, 0.90).

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nabis dependence among COGA participants. In our study, however, we found evidence for linkage to chromosome 4 with our quantitative cannabis problems phenotype but not with diagnostic cannabis dependence. This finding may also extend to other externalizing behaviors, as evidence for association between *GABRA2* and disorder of the externalizing spectrum continues to mount.[55]

Our highest LOD score with the dichotomous cannabis dependence diagnosis is on the 17qter. This region does not overlap with the linkage region identified by Stallings et al[20] for antisocial drug dependence on chromosome 17 in their study of adolescents and young adults. However, interesting candidate genes in this region include *TLK2* (tousled-like kinase 2), a serine/threonine kinase involved in chromatin assembly; *AATF* (apoptosis-antagonizing transcription factor), which is involved in gene transcription; and *PNMT* (phenylethanolamine *N*-methyltransferase), which catalyzes the critical conversion of epinephrine to norepinephrine. We are not aware of any published studies of the association between SNPs in these genes and substance-related behaviors.

Although modest, the LOD score on chromosome 6 is in a region of particular biological relevance. Our LOD score of 1.42 is 5 cM distal to the cannabinoid receptor 1 (*CNR1*) gene. The cannabinoid receptor encoded by this gene binds endogenous cannabinoids, such as anandamide, and 2-arachidonyl glycerol, and may thus be a target for exogenous cannabinoids as well.[56] The endocannabinoid system facilitates neurogenesis of hippocampal granule cells,[57] a process dependent on reelin.[58] Reelin has been suggested to be a candidate gene for schizophrenia,[59] thus providing a possible mechanism for the increased risk of psychotic disorders among cannabis users.[14,15,60]

Several limitations of our study should be considered. First, ours is a sample of adult Australians of predominantly Anglo-Celtic or Northern European ancestry, and results may not extrapolate to samples with other demographic characteristics (ie, other ethnicities where allele frequencies may vary). Although the NAG does include a Finnish cohort, because of the very low prevalence of illicit drug use in this sample, these data were not included in the analysis. Second, in keeping with *DSM-IV* criteria, our diagnostic assessment did not include the symptom cannabis-related withdrawal. Cannabis withdrawal has been shown in clinical and epidemiological studies to be an excellent indicator of cannabis use disorders[48,61-63] and should be considered for inclusion in subsequent genomic studies of substance-related behaviors. Third, NAG families were ascertained for heavy cigarette smoking and not specifically for cannabis use behaviors. Scoring these data with a community sample helped overcome the limitations of using a sample ascertained for a correlated behavior. In the absence of scored data, the possibility that our LOD scores would be specific to linkage to cannabis use behaviors in heavy cigarette smokers would be plausible. Fourth, we had very low power to detect linkage signals with the *DSM-IV* cannabis dependence phenotype. This measure of affection status codes individuals with 1 to 2 symptoms of cannabis dependence as unaffected (ie, diagnostic orphans), whereas such individuals are captured by the liability distribution of our novel factor score. This may have led to variations in LOD scores from the dichotomous and quantitative phenotypes. Finally, our LOD scores did not meet genomewide significance, and replication studies will be of significant importance in further establishing the validity of our findings. For these very reasons, we encourage future studies to consider ascertaining samples specifically for cannabis use disorders, of which there are none to our knowledge.

CONCLUSIONS

In addition to replicating previously identified genomic regions for drug dependence, we have identified a novel region on chromosome 1 (LOD 3.36) that may harbor susceptibility loci associated with cannabis use disorders. This presents an exciting step from the study of genetic variation as a latent construct to the actual identification of genomic regions. It is challenging and of great promise that our linkage finding occurs on the most gene-rich chromosome. Therefore, carefully planned fine-mapping and candidate gene studies will be necessary in the process of gene identification. The systematic identification of these genes of modest effect size, and their interactions with environmental risk and protective factors, will aid our understanding of the etiology of cannabis use disorders.

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Author Contributions: Drs Agrawal, Wang, Todd, Goate, Kaprio, Heath, Montgomery, and Madden and Ms Broms had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
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Data collection was conducted at Queensland Institute of Medical Research and the University of Helsinki; Washington University is the coordinating site and lead institution. Genotyping and data analysis are conducted at all 3 sites.

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