HHS Public Access

Author manuscript

Am J Med Genet B Neuropsychiatr Genet. Author manuscript; available in PMC 2017 May 25.

Published in final edited form as:

Am J Med Genet B Neuropsychiatr Genet. 2016 April ; 171B(3): 383–395. doi:10.1002/ajmg.b.32417.

Longitudinal Predictors of Cannabis Use and Dependence in Offspring From Families at Ultra High Risk for Alcohol Dependence and in Control Families

Shirley Y. Hill[1,*], **Bobby L. Jones**[1], **Stuart R. Steinhauer**[2], **Nicholas Zezza**[1], and **Scott Stiffler**[1]

[1]Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

[2]VA Pittsburgh Healthcare System, Pittsburgh, Pennsylvania

Abstract

Cannabis use is common among adolescents. Identification of the factors associated with continued heavy use into young adulthood and development of cannabis abuse and dependence is of considerable importance. The role of familial risk for addiction and an associated endophenotype, P300 amplitude, has not previously been related to cannabis use and dependence. A prospective longitudinal study spanning childhood and young adulthood provided the opportunity for exploring these factors, along with genetic variation, in the cannabis use behaviors of 338 young adult offspring from high and low familial risk for alcohol dependence families (ages 19–30). P300 data were collected multiple times in childhood. The association between young adult patterns of cannabis use or cannabis abuse/dependence was tested with genetic variation in the cannabinoid gene, CNR1, the ANKK1-DRD2 gene, and childhood developmental trajectories of P300. Young adult patterns of cannabis use was characterized by three patterns: (i) no use throughout; (ii) declining use from adolescence through young adulthood; and (iii) frequent use throughout. Following the low P300 trajectory in childhood predicted cannabis abuse and dependence by young adulthood. A four SNP ANKK1-DRD2 haplotype (G-G-G-C) was found to be significantly associated with the frequency of use patterns (P= 0.0008). Although CNR1 variation overall was not significantly associated with these patterns, among individuals with cannabis abuse/dependence the presence of one or both copies of the rs806368 A > G minor allele conferred a 5.4-fold increase (P= 0.003) in the likelihood that they would be in the frequent and persistent use group rather than the declining use group.

Keywords

CNR1; D2; cannabis use; cannabis abuse; cannabis dependence

[*]Correspondence to: Shirley Y. Hill, Ph.D., Department of Psychiatry, University of Pittsburgh Medical Center, 3811 O' Hara St. Pittsburgh, PA 15213. syh50@imap.pitt.edu.

Conflicts of interest: The authors have no conflicts of interest to report. Grant sponsor: NIAAA;

INTRODUCTION

Cannabis is the most widely used illicit drug reported by individuals 12 and older [Substance Abuse and Mental Health Services Administration, 2014]. The most recent report from the annual interview of 6,000 high-school student known as the Monitoring the Future report [Johnston et al., 2013] shows that by 12th grade 36% of students have used cannabis, with daily use reported by 6%. Additionally, the report notes that the perceived risk of using cannabis by high-school students fell in 2012 indicating possible future increases in use among teens.

The widespread use of cannabis especially among teens and young adults appears to present a public health concern because of increases in motor vehicle accidents [Drummer et al., 2004] and risky sexual behavior [Fernández et al., 2004] in association with cannabis use. Also, there are indications that lasting effects of cannabis use may occur with respect to brain structure and functioning. Using longitudinal data from the large-scale Dunedin cohort, Meier et al. [2012] found a decline in IQ scores from age 13 to 38 in association with persistent cannabis use along with impairment in a broad array of neuropsychological functioning. Moreover, cessation of use did not appear to restore neuropsychological functioning in adolescent onset users. Using diffusion tensor imaging methods, Zalesky et al. [2012] reported an association between cannabis use and changes in the right fimbria, a band of fibers near the hippocampus, a structure frequently linked to long-term cannabis use [Matochiket al., 2005; Medina et al., 2007a, b; Yücel et al., 2008]. Although cannabis use is widespread, not all users of cannabis are heavy users or go on to develop cannabis dependence. Currently, there is a need to identify the specific predictors of cannabis dependence.

Among the factors associated with problematic use are impulsivity either in the form of reduced inhibitory control or impulsive decision-making [Hayaki et al., 2011; Day et al., 2013]. Genetic loading for alcohol dependence (AD) through selection of families with multiple cases of AD also appears to increase the likelihood of developing a substance use disorder [Hill et al., 2008, 2011]. Family studies suggest that cannabis use, abuse, and dependence tend to cluster within families [Brook et al., 1991; Merikangas et al., 1998; Hopfer et al., 2003] as do other drug and alcohol disorders [Hill et al., 1977]. Specificity of alcohol and opioid dependence among relatives of alcohol and opioid dependent probands has been reported [Hill et al., 1977]. Similarly, relatives of alcohol dependent probands with cannabis abuse are more likely to be cannabis abusers [Bierut et al., 1998].

Previous studies have indicated that the cannabinoid receptor gene, CNR1 modulates risk for alcohol dependence [Schmidt et al., 2002] and polysubstance abuse [Zhang et al., 2004] as well as cannabis use. Also, CNR1 variation (rs2029353) has been reported to influence the potential neurotoxic effect of heavy cannabis use resulting in greater decline in hippocampus and amygdala volume [Schacht et al., 2012]. However, a meta-analysis of 13 studies reporting CNR1 associations with cannabis dependence syndromes did not find replication for two SNPs (rs1049353 and rs806379) [Benyamina et al., 2011].

An extensive literature now documents an association between the amplitude of the P300 component of the event-related potential and familial risk for alcohol dependence [Porjesz and Rangaswamy, 2007; Hill et al., 2009]. Also, there is evidence that developmental trajectories of P300 differ by familial risk [Hill et al., 1999]. Antecedent predictors of substance use disorders have been found in longitudinal studies that include variation in the P300 component of the event-related potential in which a single P300 value in childhood predicts substance use outcome [Carlson et al., 2004; Hill et al., 2009]. P300 amplitude collected in younger children appears to predict SUD outcome better than P300 at later ages [Hill et al., 2009]. Moreover, developmental trajectories of P300 based on repeated assessments in childhood have been found to predict substance use involvement [Hill et al., 2013] with those adolescents and young adults following the lower and slower trajectory pattern being more likely to develop substance use disorders. Genetic variation in the CHRM2 gene was found to be associated with these trajectories. Previously, an association between P300 amplitude and variation in the CNR1 gene has been reported in a small sample of 35 individuals for whom event-related potential recordings were obtained [Johnson et al., 1997], but repeated measures were not available to assess trajectories of P300.

Variation in the DRD2 receptor gene and the neighboring ANKK1 gene has been shown to affect the likelihood of developing a variety of substance use disorders including nicotine dependence [Gelernter et al., 2006], alcohol dependence [Hill et al., 2008], and opioid dependence [Xu et al., 2004; Clarke et al., 2014]. Cannabis use and dependence has also been studied in association with DRD2 variation. Comparison of 50 young adults with cannabis dependence and 50 controls has suggested that DRD2 variation mediated by neuroticism is associated with cannabis dependence [Jutras-Aswad et al., 2012]. Conner et al. [2005] studying 48 children of alcoholics found that those carrying the Taq1A variant of the DRD2 gene experienced their first cannabis high at an earlier age. Moreover, variation in the ANKK1 and DRD2 genes in patients receiving treatment for cocaine dependence appears to determine the effectiveness of pharmacotherapy [Spellicy et al., 2013].

In summary, the existing literature provides strong support for the cannabinoid gene, CNR1, and DRD2 or ANKK1 variation having an impact on SUD phenotypes. Familial loading for alcohol dependence also increases this risk as does following a developmental pattern of P300 amplitude in childhood and adolescence that is associated with greater disinhibition and likelihood of substance use problems.

The goal of this study was to assess the role of these factors in a sample of third generation offspring from pedigrees characterized by either ultra-high risk loading or those with an absence of familial loading for alcohol and other drug use diagnoses. As part of two longitudinal studies, 338 third generation offspring with child, adolescent, and young adult data were available. The present report concerns patterns of cannabis use during young adulthood and its relationship to genetic (DRD2, ANKK1, and CNR1) and endophenotypic (P300 trajectory class membership in childhood) variation.

MATERIALS AND METHODS

Participants

The high- and low-risk (control) offspring in this report were participants in one of two ongoing family studies (Cognitive and Personality Factors in Relatives of Alcoholics family study [CPFFS] and the Biological Risk Factors in Relatives of Alcoholic Women family study [BRFFS]). The offspring were first identified in childhood with continuing follow up into young adulthood. The high-risk families had been identified through a proband pair of same sex alcohol dependent siblings while one member of the pair was in a substance abuse treatment facility in the Pittsburgh area at the time of recruitment. Probands and all willing first-degree relatives (siblings and parents) were screened (Diagnostic Interview Schedule [DIS]) [Robins et al., 1981] for the presence of alcohol dependence (AD) and other Axis I (DSM-III) psychopathology along with screening for the presence of Feighner criteria for AD which requires symptoms in three categories of problems to meet criteria for alcohol dependence [Feighner et al., 1972].

Sister pairs were recruited for the BRFFS study while brother pairs were selected for the CPFFS study. Selection of control families was based on availability of a pair of same sex adult siblings. Volunteers and their first degree relatives were screened for Axis I psychopathology including alcohol and drug dependence using the DIS and family history information. Beginning in 1990, third-generation offspring between the ages of 8 and 18 years were followed longitudinally using multimodal assessment that included measures of child and adolescent environmental characteristics, event-related potential recordings, and structural MRIs along with annual clinical assessment using age appropriate structured psychiatric interviews. Similar measures were acquired at approximately biennial frequency in young adulthood starting at age 19.

Clinical Assessment

Each of the child/adolescent offspring between the ages of 8 and 18 years received an annual clinical assessment for DSM-III diagnoses using the children's version of the Schedule for Affective Disorders and Schizophrenia (K-SADS) [Chambers et al., 1985] administered separately to the parent and the child. Thereafter, annual clinical follow-ups included the Composite International Diagnostic Interview (CIDI) [Janca et al., 1992] to determine the presence or absence of DSM-IV diagnoses and the CIDI-Substance Abuse Module (CIDI-SAM) [Cottler et al., 1989; Compton et al., 1996] to measure the quantity, frequency, and pattern of substance use. Interrater reliability for diagnostic instruments exceeded 90%.

Event-Related Potential Recordings

Using an auditory oddball paradigm [Steinhauer and Hill, 1993], event-related potential (ERP) recordings were performed at approximately yearly intervals in childhood for the third-generation offspring who participated in the two longitudinal studies. To assess the impact of familial risk status on P300 amplitude, analyses were completed within two time frames, 8–12 years and 13–18 (Tables I and II). Differences were seen by familial risk status for only the 8–12 year group. Accordingly, P300 trajectories of childhood P300 obtained

while participants were between the ages of 8–12 years were used as possible predictors of cannabis use in young adulthood.

DNA Isolation and Genotyping

Genomic DNA was utilized from a resource extracted from whole blood or from EBV transformation and cryopreservation. PCR conditions were as described in Hill et al. [2004]. Genotyping was completed on a Biotage PSQ 96MA Pyrosequencer (Biotage AB, Uppsala, Sweden). Each polymorphism was analyzed by PCR amplification incorporating a biotinylated primer. Thermal cycling included 45 cycles at an annealing temperature of 60°C. A Biotage workstation was used to isolate the biotinylated single strand from the double strand PCR products. The isolated product was then sequenced using the complementary sequencing primer.

Quality Control

Quality control of SNP genotyping includes ongoing monitoring provided by Qiagen software. The software provides three quality categories for each SNP: pass, fail, and check. Data analysis is routinely performed for only those signals meeting the “pass” criterion. Signals that fail or are returned as needing further checking are rerun. If after three attempts the SNP does not meet the “pass” criterion, it is eliminated.

Genetic Data

A total of eight SNPs were analyzed: *CNR1*–rs806368, rs1049353, rs2023239, rs6454674; *ANKK1*–rs4938012, rs4938015, rs1800497, and *DRD2*–rs6277.

Multiple Comparisons

Pairwise trajectory group comparisons were considered significant if the *P*-value fell below the adjusted cutoff of $P = 0.05/3 = 0.017$ reflecting the three possible pairwise comparisons. We used SNPSpD [Nyholt, 2004] to calculate an effective number of SNPs to adjust the $P = 0.05$ cutoff for significance. This approach yields a Bonferroni adjusted cutoff of $P = 0.0073$ based on a calculated effective number of 6.83 SNPs. For the CNR1-ANKK1-DRD2 haplotype interaction, the adjusted cutoff value used was 0.017 as a result of adjusting for the three SNP CNR1 haplotypes.

Group-Based Trajectory Analysis

In order to model the relationship between our predictor variables and young adult cannabis use, trajectory analyses were first performed on the repeated measurements of cannabis use in young adulthood and the repeated assessments of P300 in childhood. Group-based trajectory analysis was used to determine if there was a mixture of trajectories that would characterize the observed data.

Cannabis use—Trajectory analysis of cannabis use data was restricted to those young adult offspring who had a minimum of two follow-up visits between the ages of 19 and 30, providing a total of 1085 assessments of frequency of cannabis use for 338 individuals (Tables III and IV). Analyses were conducted using *traj* in Stata [Jones et al., 2001; Jones

and Nagin, 2013], using a semi-parametric group-based approach for modeling. This approach provides clustering into groups of individual trajectories that follow similar developmental paths using a discrete mixture of appropriate parametric distributions. This group-based modeling approach provides characterization of the developmental trajectories of cannabis use based on the reported frequency of use in successive waves of assessment obtained during young adulthood. A censored normal model was used to accommodate a minimum frequency of 0 days (no use) and maximum of 7 days per week. Three models were considered (intercept only, linear, and quadratic with age). Missing recordings were assumed to be missing at random. DNA was available for 283 offspring for genotyping CNR1 and the ANKK1-DRD2 region.

Auditory event-related potential recordings and analysis of ERP—Each subject performed two blocks (80 trials each) of a Choice Reaction auditory task using high (1500 Hz) and low pitched (800 Hz) tones, presented every 3 sec (70 dB; 40 ms duration with an abrupt [2 ms] rise time) in a modified oddball paradigm in which subjects are asked to press a button (right or left) corresponding to the presence of a high or low tone as previously described [Hill et al., 1990, 1995; Steinhauer and Hill, 1993]. High-pitched tones occurred on 25% of the trials. Each trial was sampled for 1200 ms at 8 ms intervals beginning with a 200 ms prestimulus baseline. Recording conditions including stimulus presentation were held constant throughout the longitudinal study. A Grass Model 12 Neurodata system set to a bandpass of 0.01 to 30 Hz provided 20k amplification. Electrodes (Ag/AgCl) were placed at frontal, vertex, parietal, and occipital locations (Fz, Cz, Pz, Oz, P3, P4); an additional electrode placed under the left eye and referred to linked ears was used for recording eye movement and blink artifacts. All active electrodes were referred to linked ears with a forehead ground. Any trial affected by eye artifact (blinks or eye movements greater than 50 μV) were excluded online. Average ERPs were computed from artifact-free trials.

ERP analysis—A computer algorithm was used to search for the maximum peak amplitude for each component within predefined latency ranges (80–136 ms at Cz for N100, 136–240 ms at Cz for P200, 200–320 ms at Cz for N250, and 264–424 ms at Pz for P300). Peaks were verified offline by two trained raters blind to diagnosis of the subject through this interactive algorithm. The peak amplitude was computed as the deviation from the median voltage during the 200 ms prestimulus baseline.

P300 trajectories—Trajectories of childhood P300 were determined by restricting the analyses to those offspring with two or more ERP records in childhood during the specified age range (8–12 years), and who also had follow-up data available from young adulthood (N = 163 offspring). Using a mixture analysis method that allowed for determination of group placement for each individual, 467 P300 data points were modeled to determine the number of trajectories present within the sample. Group based modeling was performed using a Gaussian model. The Bayesian Information Criterion (BIC) values obtained were used as goodness of fit measures to select the number of trajectory groups for the modeling. Because individual participants do not follow an identified group trajectory path perfectly, probabilities of individual membership in each trajectory group were calculated from the model. Assignment to low, moderate, and high trajectory groups was based on the highest

probability of membership in a given category. Membership in the three groups did not differ by age at first visit or last visit, number of visits during the respective age groups (8–12 or 13–18), or by familial risk group status (Tables V and VI).

Genetic models—Additive and dominant genetic models were tested for individual SNPs with the minor allele as the effect allele. SNPs were tested for departure from Hardy-Weinberg equilibrium. No SNP was found to exhibit statistically significant HWE departure.

Genetic maps—The Genetic Map Interpolator (GMI) software [Mukhopadhyay et al., 2010] was used to retrieve current physical map positions from Ensembl (Ensembl 73). These physical positions were then used to linearly interpolate genetic map positions based on the Rutgers Combined Linkage-Physical Map [Kong et al., 2004; Matise et al., 2007].

Haplotype inference—Linkage disequilibrium (LD) analysis was performed using the HAPLOVIEW program version 4.2 [Barrett et al., 2005]. The LD structure was defined by calculating D′ values pairwise between SNPs. The offspring haplotypes were imputed using the program PHASE, version 2.1.1 [Stephens et al., 2001; Stephens and Donnelly, 2003]. The assigned haploytpes were then used in analyses as alleles in a multiallelic framework for association with the cannabis frequency groups, cannabis use and dependence groups, and P300 trajectory classes. We also considered the approach implemented in the program Unphased [Dudbridge, 2008], which incorporates the uncertainty in haplotype assignment into the test for association.

Generalized linear mixed effects modeling—Association of the cannabis use frequency trajectory groups with familial risk, candidate SNPs, and inferred haplotypes was performed using mixed effects generalized linear modeling. The model was fit using *gsem* in Stata 13 for multinomial tests which provides an estimate of the overall relationship for the three trajectory groups. Stata 13 *xtmelogit* was used to perform pair-wise comparisons and included a random effect to adjust for family relatedness for subjects with siblings included in the analysis. Genetic effects were considered individually for each SNP with haplotype analysis performed assuming an additive genetic model.

RESULTS

Group-Based Trajectory Modeling

Cannabis use—Age-related patterns of young adult cannabis use clustered into three trajectory groups: (i) a no cannabis use during young adulthood group; (ii) a declining use with age group; and (iii) a frequent use group (Fig. 1). The majority of subjects (70.4%) reported no cannabis use during young-adulthood (Table IV). The declining use group comprised 19.2% of the young adult sample. Individuals within this group reported using cannabis an average of 3.5 days/week starting at age 19 and decreasing to 1.1 days/week at age 30. The frequent use group represented 10.4% of the young adult sample. This group reported consistent, frequent use averaging 6.4 out of 7 days per week.

Individuals in the frequent use group were more often from high-risk families with multiple cases of alcohol dependence in their family background (80.0%) and were more often male

(68.6%). Among those in the group showing a decline in use during young adulthood, 60.0% were from a familial high-risk background, many of whom started using cannabis in adolescence. The no use group was disproportionately represented by individuals from the low-risk familial background (58.8%).

Cannabis trajectory group fit—The mean probabilities of membership in each of the three cannabis groups were 95.1%, 83.5%, and 90.8% for the no use, declining, and frequent use groups respectively, reflecting reasonable fit to the data. Similarity in the observation period for each group can be seen using the age at first and last visit (Table III). The trajectory groups did not differ in the percentage who were genotyped or the number of follow up visits for each subject and were of similar age (Table IV).

P300 amplitude—Three patterns of P300 development were found (Fig. 2). The 8–12 year olds were more likely to be in the lower amplitude trajectory group (60.1%) or in the moderate P300 amplitude group (35%), with only 4.9% having a development pattern in which P300 amplitude remained elevated. P300 Trajectory groups did not differ in the number of participant visits, or age at first or last visit (Tables V and VI). Females were more heavily represented in the moderate P300 group (66.7%) and males were more frequent in the low P300 group (64.3%).

P300 trajectory group fit—Because each individual was assigned a probability of being in each of the three trajectory groups, a mean probability of assignment to the group for all individuals within the group could be calculated. The mean probabilities of membership in the three P300 trajectory groups for the 8–12-year-old group were 90.1%, 84.7%, and 94.7% for the low, moderate, and high P300 groups respectively, indicating that the trajectories chosen were a good fit to the data. Characteristics of the individuals assigned to the three groups did not differ (Table V). For comparison, data for the 13–18 year old group are provided (Table VI).

Familial risk and cannabis trajectories—Membership in the three cannabis use groups was highly related to familial risk status (Table VII). High-risk individuals were 11.8 times more likely to be in the frequent use group ($P = 0.001$, 95%CI = [2.6, 53.4]) than in the no use group. Also, the high-risk subjects were 2.4 times more likely to be in the declining use with age group ($P = 0.007$, 95% CI = [1.3, 4.6]) than the no use group, possibly reflecting a higher use in adolescence and the earlier years of young adulthood than their low-risk counterparts.

Cannabinoid receptor gene and cannabis trajectories—The CNR1 SNPs individually were not associated with membership in the three trajectory groups derived from the cannabis frequency data. However, among those who had received an abuse or cannabis dependence diagnosis, subjects with either one or both copies of the CNR1 rs806368 A > G minor allele were 5.4 times more likely to be in the frequent use trajectory group than in the declining use with age group ($P = 0.003$, 95%CI = [1.8, 16.8]).

P300 trajectory class and cannabis abuse or dependence—Among male participants, membership in the low P300 developmental trajectory class in early childhood

was associated with having a young adult diagnosis of cannabis abuse or dependence. Nineteen of 63 male subjects with low P300 development in childhood were found to have a cannabis abuse or dependence diagnosis in comparison to 1 of 23 males with membership in the moderate or higher P300 amplitude trajectory classes, $P = 0.01$. Males with high familial risk were more likely to follow the lower amplitude P300 developmental trajectory pattern (OR = 4.0, $P = 0.010$; 95%CI = [1.4,11.6]). A relationship between P300 trajectory class and development of cannabis abuse or dependence was not seen in female participants.

Cannabinoid SNP variation and cannabis abuse or dependence—Each of the four CNR1 SNPs (rs806368, rs1049353, rs2023239, rs6454674) and the four ANKK1-DRD2 SNPs (rs4938012, rs4938015, rs1800497, rs6277) were individually tested for their association with presence or absence of a diagnosis of either cannabis abuse or dependence during the longitudinal follow up period. Only one SNP showed nominal significance, rs1049353, with a *P*-value of 0.03 in a test for the presence of any minor allele, and marginal significance ($P = 0.06$) under an additive model.

Frequency of use and ANKK1-DRD2 haplotypes—The pair-wise linkage disequilibrium between SNPs and the LD block structure are shown in Figure 3 with the results summarized in Table VIII. The four-SNP *ANKK1–DRD2* haplotype block, rs4938012–rs4938015–rs1800497–rs6277 was found to be associated with membership in the young adult cannabis use trajectory classes (Phase/GLMM overall $P = 0.007$). Compared to the no use group, those with any copy of the haplotype G-G-G-C were 2.9 times more likely to be in the frequent use or declining use with age groups (95%CI=[1.5, 5.6], Unphased $P=0.00003$, Phase/GLMM $P = 0.0008$).

Frequency of use, cannabis abuse or dependence, and the interaction of ANKK1-DRD2 and CNR1—Haplotypes were formed for the ANKK1-DRD2 SNPs (Fig. 3) and the CNR1 SNPs (Fig. 4). Significant interactions were found in comparisons of users and nonusers for three of the DRD2 haplotypes when interacted with the two SNP CNR1 haplotypes (Table IX).

CNR1, ANKK1-DRD2, SNP variation and P300 trajectory class membership—Each of the four CNR1 SNPs and the four ANKK1-DRD2 SNPs were individually tested for their association with childhood P300 trajectory class membership. None of the individual SNPs were related to P300 trajectory class membership. However, within the high-risk group one SNP, rs806368, showed a *P*-value of 0.007 in a test for the presence of any minor allele such that those without a minor allele were significantly more likely to be in the lower amplitude P300 trajectory class.

DISCUSSION

The addictive properties of cannabis have been linked to CB1 cannabinoid receptors based on animal studies in which CB1 antagonists were administered, or mice deficient in CB1 receptors were studied [Maldonado and Rodríguez, 2002]. Human studies have investigated a number of polymorphisms within the CB1 receptor gene localized to Chromosome 6q14–q15, finding an association between cannabis dependence and CNR1 variation. Of the seven

SNPs previously reported to be associated with cannabis dependence or substance use disorder (rs806368, rs806379, rs806380, rs1049353, rs2023239, rs6454674, and rs12720071), the present investigation included four (rs806368, rs1049353, rs2023239, rs6454674). These SNPs provide moderate support for an association between CNR1 and cannabis abuse/dependence.

A highly significant relationship between one of the ANKK1-DRD2 haplotypes and membership in the combined frequent use or frequent with declining use classes versus no use trajectory class was seen. Although none of the CNR1 SNPs alone predicted membership in cannabis trajectory classes, we did see a relationship between the interaction of CNR1 and ANKK1-DRD2 haplotypes and whether the subject was in the combined frequent use or frequent with declining use classes versus the no use class.

Although there is substantial agreement that the CNR1 gene is involved in cannabis dependence and other substance dependence, a significant amount of inconsistency has been reported for specific SNPs. A meta-analysis and review of 11 association studies of CNR1 rs1049353, rs806379, and a microsatellite AAT and substance dependence concluded that only the AAT repeat showed only a modest association [Benyamina et al., 2011]. Others have noted that rs2023239 is a key player in cannabis dependence and other SUDs because of its affect on CB1 expression. This SNP located in exon three encodes an A–G substitution resulting in an alternative splice variant [Zhang et al., 2004]. The rs2023239 G allele has been demonstrated to be associated with greater CB1 expression in postmortem prefrontal cortex [Hutchison et al., 2008] and greater withdrawal and craving in recently abstinent cannabis users [Haughey et al., 2008]. Variation in rs2023239 also appears to influence brain response to cannabis cues with greater activation in reward circuitry including the orbitofrontal cortex, anterior cingulate gyrus, and nucleus accumbens [Filbey et al., 2010]. Additionally, cannabis use has been reported to decrease volume of the amygdala and hippocampus in association with rs2023239 variation [Schacht et al., 2012]. The present report finds a haplotype including this SNP (rs2023239-rs6454674 T-C haplotype) interacting with ANKK1-DRD2 significantly associated with frequency of cannabis use (frequent or frequent use that declines with age versus no use). This interaction was also associated with the presence of cannabis abuse or dependence. In short, variation in the rs2023239 SNP of the CNR1 gene confers changes in level of cannabis use and likelihood of dependence, and appears to affect volume and functionality of specific brain regions.

The process of developing cannabis dependence undoubtedly involves many stages in which the individual transitions from anon user to user, followed by increased frequency of use and dependence. In a review of twin studies, Agarwal and Lynskey [2006] suggest that each stage of the process is influenced by heritable factors, with later stages that include cannabis dependence being more influenced by genetic factors than are earlier stages. The present results support an association between CNR1 in users meeting criteria for abuse or dependence. However, the relationship was not present in all cannabis users suggesting that CNR1 variation may not be instrumental in influencing behavior in the early stages of cannabis use.

Also, it has been suggested that the observed greater heritability in later stages may be due to confounding of genetic factors that are shared across use, abuse and dependence and not specific to cannabis abuse/dependence [Agarwal and Lynskey, 2006]. Congruently, we find that dopaminergic mediation which is common to other abuse/dependence syndromes such as alcohol, opioid, or nictoine dependence appears to contribute to this increase in genetic mediation in later stages of cannabis use leading to development of cannabis abuse/dependence. Findings for the ANKK1-DRD2 haplotype analysis are of particular interest because of the association found between frequency of cannabis use and variation within this gene complex. Unlike the findings for CNR1, a relationship between ANKK1-DRD2 variation and frequency of use was present beyond the group of cannabis users who developed problems sufficient to meet DSM-IV criteria for cannabis abuse or dependence. Interestingly, when variation in both the CNR1 haplotype and ANKK1-DRD2 haplotype were tested together an association was also seen for both the frequency of use phenotype and the cannabis abuse/dependence phenotype.

A variety of mechanisms appear to participate in the addictive properties of the cannabinoids. Cannabinoids increase the firing rate of dopaminergic neurons in the ventral tegmental area (VTA) similar to that seen for other drugs of abuse [DiChiara et al., 2004]. However, one hypothesis is that cannabinoids may only indirectly activate mesolimbic dopaminergic pathways through its effects on the functional balance between inhibitory effects of GABA ergic interneurons and glutaminergic excitatory effects primarily from the prefrontal cortex [Maldonado and Rodríguez, 2002; Maldonado et al., 2011]. Other support for dopamingergic modulation of cannabinoid effects comes from data showing that D2 receptor agonists increase the discriminative effects of low doses of tetrahydracannabinol (THC) [Solinas et al., 2010]. Additionally, withdrawal from chronic THC administration is accompanied by reduced dopaminergic transmission in the limbic system [Diana et al., 1998].

The present study supports previous findings from our laboratory suggesting that developmental trajectories of P300 amplitude are associated with familial risk for alcohol dependence [Hill et al., 1999], incidence of childhood psychiatric disorders [Hill and Shen, 2002], and later development of substance use disorders [Hill et al., 2009, 2013]. This is the first demonstration that P300 trajectories in childhood are associated with cannabis dependence outcome by young adulthood though the relationship appeared to be specific to male participants. There are a number of possibilities why this relationship may have been found. Cannabis use and dependence occurs more frequently among individuals who score higher on self report measures of impulsivity [van Leeuwen et al., 2011]. Moreover, lower P300 amplitude has been shown to be related to a variety of behaviors that comprise a broad externalizing disorder spectrum that is associated with alcohol and drug dependence, nicotine dependence, conduct disorder, and adult antisocial disorder all involving impaired impulse control [Patrick et al., 2006]. Alternatively, CNR1 or DRD2 variation may have a direct effect on P300 amplitude that is related to cannabis use outcome. Variation in the CNR1 gene (a microsatellite AAT repeat) has been reported to be associated with P300 amplitude in a sample of 35 individuals [Johnson et al., 1997]. Also, acute doses of Δ^9-THC in association with AAT variation has been shown to reduce P300 amplitude and prolong P300 latency [Stadelmann et al., 2011]. Similarly, two studies have shown a relationship

between the DRD2 Taq1A allele and P300 amplitude [Hill et al., 1998; Anokhin et al., 1999]. The Taq1A variant was subsequently localized to the ANKK1 gene, a gene involved in signal transduction pathways [Neville et al., 2004]. The Taq1A causes an amino acid substitution in the 11th ankyrin repeat of ANKK1. The ANKK1 gene lies in close proximity to the DRD2 region and appears to have a role in DRD2 functioning. The Taq1A variation has been extensively studied for possible association with a variety of neurological and psychiatric conditions [Noble, 2003]. Early studies used restriction fragment length polymorphism (RLFP) technology to genotype Taq1A variation but it is now studied using SNP rs1800497 as was done in the present study.

It is uncertain why a relationship between P300 childhood trajectory class membership and ANKK1-DRD2 variation was not found in the present study in view of previous reported associations with the Taq1 A allele. Only moderate support for CNR1 variation having a direct effect on trajectories of P300 amplitude was seen with the effect seen only in those at high familial risk for alcohol dependence. Because both CNR1 and ANKK1-DRD2 variation were associated with cannabis use behaviors, but only CNR1 had a direct effect on P300 amplitude, it would appear that each are independent predictors of cannabis use young adult outcome.

Some limitations of the present analyses should be mentioned. Although several neurotransmitters appear to mediate the relationship between cannabis use and enhanced firing of dopaminergic neurons in the VTA (GABA, glutamate, acetylcholine, corticotrophin-releasing factor) (see Maldonado et al. [2011]), the present report only addressed the influence of the ANKK1-DRD2 genes.

Another limitation is the reliance on frequency of use to define patterns of adult cannabis use. With the wide variation in the content of THC in cannabis purchased from the street, it is uncertain if frequency of use can be a surrogate variable for dose levels received over time. A study contracted by the National Institute of Drug Abuse (NIDA) has examined confiscated marihuana from 1993 to 2008 by law enforcement finding an increase in the THC content from 3.4% in 1993 to 8.8% in 2008 [Mehmedic et al., 2010]. Because the potency of cannabis available today appears to exceed that which was available 10–20 years ago when these participants began using cannabis, decreased frequency may also have been accompanied by either maintained or increased dosage. Nevertheless, frequency of use does appear to be highly related to the likelihood that development of cannabis abuse and dependence will occur, and provides a useful index for determining which individuals are most vulnerable to developing abuse and dependence.

The relationship between P300 trajectories in childhood and young adult cannabis abuse and dependence was demonstrated in sample consisting of both ultra high-risk and low- risk controls. This raises the question of whether such a relationship would be uncovered in an unselected population-based sample. While demonstration within a highly selected sample might be viewed as a limitation, there is reason to believe that this type of sample provides an efficacious means for identifying significant predictor variables that can then be tested in large-scale population-based studies. Uncovering the public health implications for the population at large regarding potential adverse outcomes of widespread use of cannabis

appear to benefit from both strategies. Studies demonstrating reduced P300 in offspring of alcohol dependent parents including the now classic studies [Begleiter et al., 1984; Hill et al., 1990; Hill and Steinhauer, 1993] have shown replication in population-based studies [Carlson et al., 2004]. However, one important way these studies have differed in their findings from those seen in ultra high- risk families is that the population-based studies have also shown reduction of P300 in adolescent and young adult individuals with reduced amplitude of P300 that predicts substance use outcome. One explanation for these differences may be based on the lesser severity and later onset of substance use problems seen in samples drawn from the general population than is seen in ultra high-risk samples where selection criteria leads to greater familial/genetic loading [O'Brien et al., 2014]. Later onset externalizing disorders seen in population samples may also mean that those showing such disorders may have reduction in P300 at a later point in time, namely, during adolescence. Ultra high-risk samples with earlier onset for development of substance use disorders show reduced amplitude of P300 in childhood (ages 8–12) but additionally show a trajectory pattern across childhood and adolescence suggesting a developmental delay in achieving age appropriate P300 and as consequence appear to "catch up" with controls by adolescence [Hill et al., 1999].

Although there are limitations to the analyses presented, there are a number of strengths the data set has offered. First, prior to the present study there were no follow ups spanning childhood, adolescence, and young adulthood in which cannabis use behaviors have been recorded that have included the P300 endophenotype, familial risk assessment, and genetic variation in CNR1 and ANKK1-DRD2 as potential predictors of cannabis use or abuse/dependence. Second, because both cannabinoid and dopaminergic variation were assessed within the same set of individuals, the effects of each alone and as interactive effects could be determined. Finally, the present set of analyses provides an important delineation between biomarkers of cannabis initiation and biomarkers of abuse/dependence.

Acknowledgments

We wish to acknowledge the contribution of the family members who have contributed DNA, participated in multiple interview sessions over several years, and continue to be involved in clinical follow up. Also, we want to acknowledge the contributions of many clinical staff in assessing research participants to determine phenotypic data. A special thanks to Brian Holmes for assistance in manuscript preparation and Jeannette Locke-Wellman for database support. The financial support for the study reported in this manuscript was provided by NIAAA Grants AA018289, AA05909, AA08082, and AA015168 to SYH.

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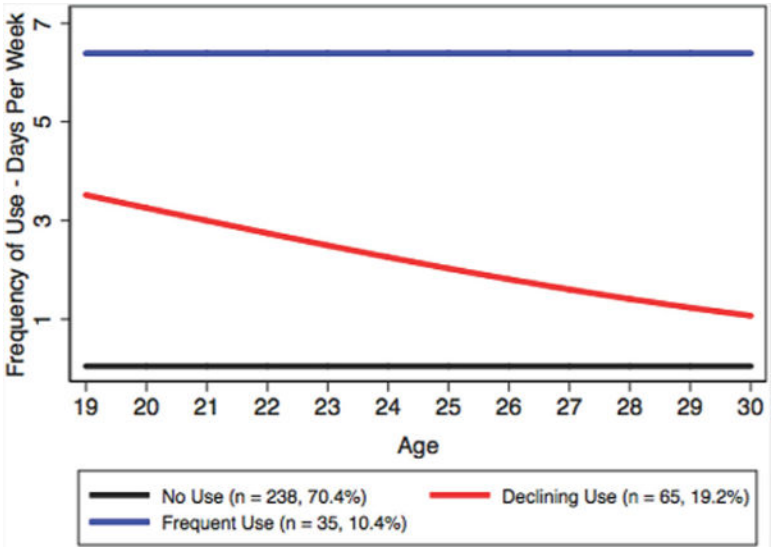

FIG. 1.

Trajectory models for cannabis use frequency by age. Three trajectory classes characterize individual use between the ages of 19 and 30. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

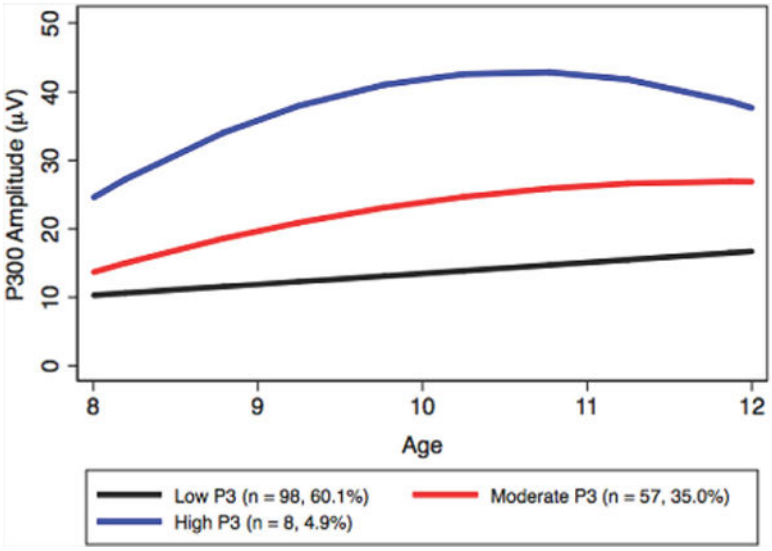

FIG. 2.

Trajectory models for P300 amplitude by age. P300 was obtained from the midline parietal scalp location to infrequent target stimuli. Three trajectory classes characterize individuals between the ages of 8 and 12. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

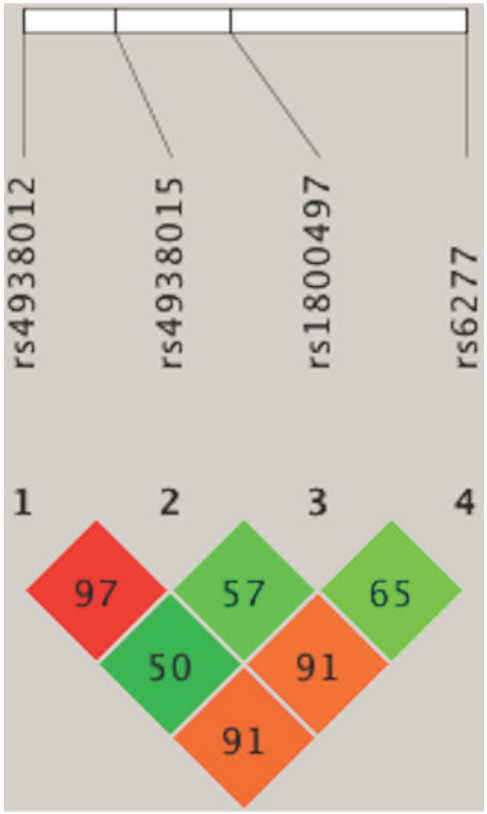

FIG. 3.

ANKK1-DRD2 linkage disequilibrium display from HAPLOVIEW. The four-SNP block showed a significant relationship with cannabis use trajectories. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

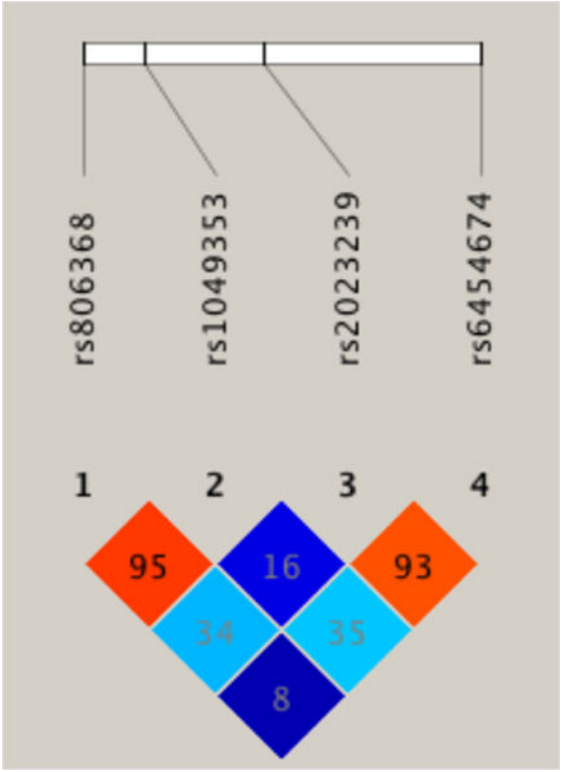

FIG. 4.

CNR1 linkage disequilibrium display from HAPLOVIEW. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmgb].

TABLE I

Sample Characteristics by Sex and Familial-Risk for 163 Subjects Ages 8–12

	Male		Female		*P*-value		
Familial risk	**High**	**Low**	**High**	**Low**	**Sex**	**Risk**	**Sex × risk**
Subjects (% of 163 total)	43 (26.4%)	43 (26.4%)	43 (26.4%)	34 (20.9%)	NA	NA	NA
Subject visits (% of 467 in 8–12)	122 (26.1%)	119 (25.5%)	128 (27.4%)	98 (21.0%)	NA	NA	NA
Visits per subject in 8–12	2.8 ± 1.0	2.8 ± 0.9	3.0 ± 0.9	2.9 ± 1.0	NS	NS	NS
Age at first visit (8–12)	9.2 ± 1.0	9.4 ± 1.0	9.3 ± 0.9	9.1 ± 0.9	NS	NS	NS
Age at last visit (8–12)	11.8 ± 0.8	11.8 ± 0.6	11.8 ± 0.6	11.6 ± 0.8	NS	NS	NS
P300 amplitude (μV) at first visit (8–12)	13.9 ± 7.9	17.3 ± 10.4	17.7 ± 10.5	15.8 ± 8.9	0.05	0.06	0.07
P300 amplitude (μV) at last visit (8–12)	17.5 ± 7.2	21.4 ± 9.8	24.8 ± 8.9	21.5 ± 9.2	<0.001	0.006	0.007
SES category[a]	3.5 ± 1.0	3.9 ± 1.0	3.2 ± 1.0	4.0 ± 07	NS	0.007	NS

[a]Socioeconomic Status (SES) was calculated using occupation and education as previously described in Hill et al. [2008].

TABLE II

Sample Characteristics by Sex and Familial-Risk for 321 Subjects Ages 13–18

	Male		Female		*P*-value		
Familial risk	**High**	**Low**	**High**	**Low**	**Sex**	**Risk**	**Sex × risk**
Subjects (% of 321 total)	78 (24.3%)	89 (27.7%)	88 (27.4%)	66 (20.6%)	NA	NA	NA
Subject visits (% of 1313 in 13–18)	329 (25.1%)	388 (29.5%)	333 (25.4%)	263 (20.0%)	NA	NA	NA
Visits per subject (13–18)	4.2 ± 1.5	4.4 ± 1.4	3.8 ± 1.4	4.0 ± 1.4	NS	NS	NS
Age at first visit (13–18)	14.1 ± 1.0	13.9 ± 0.9	14.2 ± 1.0	14.2 ± 1.0	NS	NS	NS
Age at last visit (13–18)	17.9 ± 1.1	17.8 ± 1.0	17.8 ± 1.0	17.9 ± 1.2	NS	NS	NS
P300 amplitude (μV) at first visit (13–18)	20.7 ± 8.2	20.7 ± 9.0	22.9 ± 8.5	21.1 ± 8.9	NS	NS	NS
P300 amplitude (μV) at last visit (13–18)	19.4 ± 7.5	19.6 ± 8.5	22.4 ± 8.6	21.9 ± 8.0	0.005	NS	NS
SES category[a]	3.4 ± 1.0	3.8 ± 1.0	3.4 ± 1.1	4.0 ± 0.8	NS	0.004	NS

[a]Socioeconomic Status (SES) was calculated using occupation and education as previously described in Hill et al. [2008].

TABLE III

Sample Characteristics by Sex and Familial-Risk for 338 Subjects Seen in Young Adulthood (YA, 19–30)

	Male		Female		*P*-value		
Familial risk	**High**	**Low**	**High**	**Low**	**Sex**	**Risk**	**Sex × risk**
Subjects (% of 338 total)	68 (20.1%)	94 (27.8%)	97 (28.7%)	79 (23.4%)	NA	NA	NA
Subject visits (% of 1085)	224 (20.6%)	295 (27.2%)	322 (29.7%)	244 (22.5%)	NA	NA	NA
Genotyped	64 (94.1%)	79 (84.0%)	87 (89.7%)	53 (67.1%)	0.006	<0.001	NS
YA visits per subject	3.3 ± 1.2	3.1 ± 1.0	3.3 ± 1.2	3.1 ± 1.1	NS	NS	NS
Age at first YA visit	20.5 ± 1.9	20.6 ± 2.2	21.0 ± 2.4	20.9 ± 2.4	NS	NS	NS
Age at last YA visit	26.2 ± 2.9	25.5 ± 3.1	26.6 ± 2.8	26.1 ± 3.1	NS	NS	NS
Cannabis use at first YA visit (days/week)	2.2 ± 3.1	0.4 ± 1.5	1.5 ± 2.6	0.9 ± 2.1	0.001	<0.001	0.011
Cannabis use at last YA visit (days/week)	2.4 ± 3.2	0.6 ± 1.8	1.0 ± 2.2	07 ± 2.0	<0.001	<0.001	<0.001
SES category[a]	3.3 ± 1.0	3.8 ± 0.9	3.3 ± 1.1	4.1 ± 0.9	NS	<0.001	NS

[a]Socioeconomic Status (SES) was calculated using occupation and education as previously described in Hill et al. [2008].

TABLE IV

Trajectory Group Characteristics for Cannabis Use in Young Adulthood (YA, 19–30)

	No use	Declining with age	Frequent use	*P*-value
Group size (% of 338 total)	238 (70.4%)	65 (19.2%)	35 (10.4%)	NA
High familial risk	98 (46.6%)	39 (60.0%)	28 (80.0%)	<0.001
Sex, female	127 (53.4%)	38 (58.5%)	11 (31.4%)	0.027
Genotyped (% of 283 total)	82.4%	83.1%	94.3%	NS
YA visits per subject	3.2 ± 1.1	3.2 ± 1.2	3.4 ± 1.1	NS
Age at first YA visit	20.9 ± 2.3	20.6 ± 2.2	20.5 ± 17	NS
Age at last YA visit	26.1 ± 3.0	26.2 ± 3.0	26.1 ± 3.0	NS

TABLE V

Trajectory Group Characteristics for P300 Amplitude in Childhood (8–12)

	Low	Moderate	High	*P*-value
Group size (% of 163 total)	98 (60.1%)	57 (35.0%)	8 (4.9%)	NA
High familial risk	54 (55.1%)	27 (47.4%)	5 (62.5%)	NS
Sex, female	35 (35.7%)	38 (66.7%)	4 (50.0%)	0.001
Visits per subject in 8–12	2.8 ± 0.9	2.9 ± 1.0	2.6 ± 07	NS
Age at first visit (8–12)	9.3 ± 0.9	9.2 ± 0.9	9.3 ± 0.9	NS
Age at last visit (8–12)	11.8 ± 07	117 ± 0.8	11.8 ± 07	NS
P300 at first visit (μV)	12.2 ± 6.5	20.0 ± 8.3	37.8 ± 10.9	<0.001
P300 at last visit (μV)	16.1 ± 5.9	27.5 ± 5.6	40.6 ± 8.9	<0.001
Cannabis abuse/dependence	21 (21.4%)	9 (15.8%)	0 (0.0%)	NS

TABLE VI

Trajectory Group Characteristics for P300 Amplitude in Adolescence (13–18)

	Low	Moderate	High	Highest	*P*-value
Group size (% of 321 total)	79 (24.6%)	163 (50.8%)	74 (23.0%)	5 (1.6%)	NA
High familial risk	42 (53.2%)	83 (50.9%)	39 (52.7%)	2 (40.0%)	NS
Sex, female	31 (39.2%)	77 (47.2%)	43 (58.1%)	3 (60.0%)	NS
Visits per subject in 13–18	3.8 ± 1.4	4.1 ± 1.4	4.4 ± 1.4	3.6 ± 1.3	NS
Age at first visit (13–18)	14.3 ± 1.1	14.1 ± 1.0	13.9 ± 0.9	14.0 ± 0.8	NS
Age at last visit (13–18)	*17.7* ± 1.1	17.9 ± 1.0	17.9 ± 1.0	17.1 ± 2.0	NS
P300 at first visit (μV)	12.2 ± 4.8	21.1 ± 5.1	30.5 ± 6.0	43.2 ± *7.4*	<0.001
P300 at last visit (μV)	12.0 ± 4.5	207 ± 4.8	28.9 ± 5.6	44.8 ± *7.9*	<0.001
Cannabis abuse/dependence	14 (17.7%)	39 (23.9%)	15 (20.3%)	0 (0.0%)	NS

TABLE VII

Odds of Cannabis Use Trajectory Group Membership Predicted by High Familial Risk Relative to Comparison Groups

	No use		Use declining with age		Frequent use	
	OR [95%CI]	***P***	**OR [95%CI]**	***P***	**OR [95%CI]**	***P***
High-risk versus low-risk						
No use	—		2.4 [1.3, 4.6]	0.007	11.8 [2.6, 53.4]	0.001
Declining	0.4 [0.2, 0.8]	0.007	—		4.9 [1.1, 21.2]	0.03
Frequent	0.1 [0.0, 0.4]	0.001	0.2 [0.0, 0.9]	0.03	—	

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TABLE VIII

ANKK1–DRD2 Haplotype Analysis for Frequency of Cannabis Use Trajectory Groups and Abuse or Dependence Diagnosis

		Frequency of use comparison		Cannabis abuse/dependence	
		Phase/GLMM	Unphased	Phase/GLMM	Unphased
		Any use trajectories versus no use trajectory	Any use trajectories versus no use trajectory	Any cannabis abuse or dependence	Any cannabis abuse or dependence
Markers	**Haplotype**	***P*-value[a]**	***P*-value[a]**	***P*-value[b]**	***P*-value[b]**
rs4938012-rs4938015-	G-G-G-T	0.072	0.10	ns	ns
rs1800497-rs6277	A-A-G-C	ns	ns	ns	ns
(ANKK1–DRD2)	G-G-G-C	0.0008	0.00003	0.027	0.018
	A-A-A-C	ns	ns	ns	ns

[a]Comparing combined frequent use and declining with age use trajectory groups to the no use group.

[b]Association with cannabis abuse/dependence.

TABLE IX

CNR1 2 SNP Haplotype Interactions With ANKK1–DRD2 Haplotypes: Analysis of Frequency of Cannabis Use Trajectory Classes and Cannabis Abuse or Dependence Diagnosis

				Phase/GLMM	Phase/GLMM
ANKK1–DRD2	**ANKK1–DRD2**	**CNR1**	**Interacting withCNR1**	**Any use trajectories versus no use trajectory**	**Any cannabis abuse or dependence**
Markers	**Haplotype**	**Markers**	**Haplotype**	***P*-value[a]**	***P*-value[b]**
rs4938012-rs4938015-rs1800497-rs6277	G-G-G-T	rs806368-rs1049353	G-G	0.018	ns
	A-A-G-C	rs2023239-rs6454674	T-C	0.030	0.033
	G-G-G-C	rs2023239-rs6454674	C-A	0.065	ns
	A-A-A-C	rs806368-rs1049353	A-A	0.037	ns

[a]Comparing combined frequent use and declining with age use trajectory groups to the no use group.

[b]Association with cannabis abuse or dependence.