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Lifetime use of cannabis from longitudinal assessments, cannabinoid receptor (CNR1) variation, and reduced volume of the right anterior cingulate

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Abstract

Lifetime measures of cannabis use and co-occurring exposures were obtained from a longitudinal cohort followed an average of 13 years at the time they received a structural MRI scan. MRI scans were analyzed for 88 participants (mean age=25.9 years), 34 of whom were regular users of cannabis. Whole brain voxel based morphometry analyses (SPM8) were conducted using 50 voxel clusters at $p=0.005$. Controlling for age, familial risk, and gender, we found reduced volume in Regular Users compared to Non-Users, in the lingual gyrus, anterior cingulum (right and left), and the rolandic operculum (right). The right anterior cingulum reached family-wise error statistical significance at $p=0.001$, controlling for personal lifetime use of alcohol and cigarettes and any prenatal exposures. CNR1 haplotypes were formed from four CNR1 SNPs (rs806368, rs1049353, rs2023239, and rs6454674) and tested with level of cannabis exposure to assess their interactive effects on the lingual gyrus, cingulum (right and left) and rolandic operculum, regions showing cannabis exposure effects in the SPM8 analyses. These analyses used mixed model analyses (SPSS) to control for multiple potentially confounding variables. Level of cannabis exposure was associated with decreased volume of the right anterior cingulum and showed interaction effects with haplotype variation.

1. Introduction

The 2013 National Survey on Drug Use and Health (NSDUH) (SAMHSA 2014) reported that marijuana is the most commonly used illicit drug in persons age 12 or older with 19.8 million individuals in the US reporting use in the past month. Daily use climbed from 5.1 million persons in 2007 to 8.1 million in 2013. The Monitoring the Future survey (Johnston et al., 2013) revealed that by 12th grade 36% of students report using marijuana in the past month, with daily use reported by 6%. A number of consequences of marijuana use present public health concerns. Of particular importance is the possible lasting effects on brain structure and functioning that occur in association with marijuana use. Despite evidence pointing to health problems in association with long-term cannabis use (Murray et al., 2007; Hall and Solowij, 1998), the dose-dependent effects of cannabis use on brain morphology have been assessed in only a few human studies. Moreover, documenting the relationship

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between cannabis use and brain alteration has been challenging because cannabis users tend to be users of other commonly used drugs such as alcohol and cigarettes.

The heritability of ever using cannabis or becoming dependent on it is moderately high (between 17–76%), suggesting that cannabis use behaviors have genetic underpinnings (Agarwal and Lynskey, 2006 for review). There are two known receptors within the endocannabinoid system. The cannabinoid receptor 1 (Human Gene Nomenclature Committee [HUGO approved symbol CNR1]) and alternatively known as CB1, has a cytogenetic location at chromosome 6q15 and is found throughout the brain where it is highly expressed (Childers and Breivogel, 1998) while the cannabinoid receptor 2 (CNR2), also known as CB2 appears to be confined to the periphery. Type I cannabinoid receptors have been localized to multiple brain regions including the prefrontal cortex, amygdala, hippocampus, striatum, and cerebellum (Herkenham et al., 1991; Eggen and Lewis, 2007). The cannabinoid gene (CNR1) encodes for CB1 receptor making it an ideal candidate gene for studying cannabis exposure effects.

Cannabis use has been associated with reduction of volume in a number of brain regions, particularly those rich in cannabinoid CB1 receptors, including the medial temporal cortex, parhippocampal gyrus (Matochik et al., 2005), insula and orbitofrontal cortex (Battistella et al., 2014, Filbey et al., 2014), hippocampus and amygdala (Yücel et al., 2008; Schacht et al., 2012; Lorenzetti et al., 2015; Cousijn et al., 2012), and cingulum (Bangalore et al., 2008; Rapp et al., 2013) with users showing reduced volume relative to non-users. Although results of studies assessing the effects of cannabis on brain structure have been mixed, what is clear is that regions that appear to show reduction in volume in association with cannabis use are regions that have an abundance of CB1 receptors. Although variation in the CNR1 gene appears to confer important variation that should be considered in understanding the effects of cannabis exposure, only a few studies to date have investigated this relationship. Schacht et al. (2012) showed that cannabis users had smaller bilateral hippocampi and amygdale in association with variation in the G allele of rs2023239 of the CNR1 gene. Similarly, CNR1 genetic polymorphisms along with marihuana use have been found to be associated with white matter changes and cognitive deficits in participants with schizophrenia (Ho et al., 2011).

In spite of multiple reports of reduced volume of brain regions in association with cannabis use, some have questioned whether the gray matter loss seen can be attributed to cannabis use per se because individuals who use cannabis frequently use other drugs. One recent report found that cannabis use effects were not found when alcohol use was controlled for in statistical analyses (Weiland et al., 2015). In a large study of 483 participants, half of whom were exposed to cannabis, Pagliaccio et al. (2015), reported that cannabis exposure was significantly related to reduction in left amygdala (2.3%) and ventral striatal (3.5%) volume. However, the authors interpret their findings as casting doubt on the assumption that cannabis is the causative agent in this volume reduction. Their conclusion was based on an analysis of 89 sibling pairs discordant for cannabis exposure who showed similar amygdala volume suggesting that genetic relatedness between siblings provide greater explanatory variance than did cannabis exposure.

The present report investigated the effects of lifetime cannabis use on brain morphology while controlling for lifetime use of alcohol and cigarettes in individuals followed longitudinally through adolescence and young adulthood. Possible effects of maternal use of alcohol and cigarettes and familial loading for alcohol dependence were also explored to insure that effects found for personal use of cannabis were not due to these sources of variation. Importantly, all cannabis exposure effects were evaluated as possible interactions with CNR1 variation.

2. Methods

2.1. Participants

The present report is based on structural MRI scans of 107 offspring followed through childhood, adolescence and young adulthood who are part of an ongoing family study. The family study included multiplex for alcohol dependence families and control families.

2.1.1. Multiplex for alcohol dependence (AD) families—The multiplex for alcohol dependence families were identified through an adult proband pair of alcohol dependent sisters as previously described (Hill et al., 2011). The sisters were screened using an in-person structured interview (Diagnostic Interview Schedule) (DIS; Robins et al., 1981) to determine the presence of alcohol dependence and other Axis I psychopathology. The DIS interview was administered to all available first-degree relatives (other siblings and parents of the proband pair). Unavailable or deceased relatives were diagnosed by family history report. Families were excluded if the proband or her first-degree relatives met criteria for primary recurrent major depression, bipolar disorder, primary drug dependence (i.e. drug dependence preceded alcohol dependence by 1 or more years) or schizophrenia. Available spouses were diagnosed using the same methods as members of the target families.

2.1.2. Low risk control families—Each control family was selected on basis of residence within a census tract from which a high-risk family had been selected, with an attempt made to yoke each control family to a high-risk family in the study. An adult woman was designated as the “index” case and screened for absence of alcohol and drug dependence using the DIS, though other psychopathology was free to vary. Diagnostic data were also collected for all first degree relatives using either an in-person DIS interview or by obtaining multiple family history reports to ensure an absence of a family history of alcohol dependence. Accordingly, all control families had a low density of familial alcohol dependence.

The goal of the larger longitudinal study is to investigate clinical and neurobiological characteristics of offspring from high and low-familial risk for alcohol dependence. To achieve this end, offspring were followed through childhood at approximately annual intervals and through young adulthood, biennially. These follow-up visits provided extensive assessment of alcohol and drug use information obtained at each follow-up wave. All participants provided consent with each visit. Children provided assent with parental consent. The study has ongoing approval from the University of Pittsburgh Institutional Review Board.

2.2. Longitudinal clinical assessment of children, adolescents and young adults

All available offspring from high and low-risk for alcohol dependence families who were 8–18 years old at the initiation of the follow up were eligible for inclusion. Control offspring were selected through parents who were screened for absence of alcohol and drug dependence using the DIS. The study plan included yearly assessment of offspring between the ages of 8–18 years of age and biennial assessment of young adults following their 18th birthday.

2.3. Assessment of personal use of cannabis and other substances

2.3.1. Child assessment—Each child/adolescent and his/her parent were separately administered the Schedule for Affective Disorders and Schizophrenia (K-SADS) (Chambers et al., 1985) by trained, Masters' level clinical interviewers with a follow-up interview performed by an advanced resident in child psychiatry at each annual evaluation. A reliable best-estimate diagnosis was obtained for all major DSM-III diagnoses including drug and alcohol abuse and dependence at approximately yearly intervals (Hill et al., 2011). Quantity and frequency of use of cannabis and other substances was also obtained. In accordance with KSADS administration requirements, all information was acquired by asking the participant and his/her parent to respond with reference to the past year.

2.3.2. Young adult assessment—Young adult assessment included administration of the Composite International Diagnostic Interview (CIDI) (Janca et al., 1992) which covers symptoms needed to meet all DSM-IV criteria including cannabis abuse and dependence. The CIDI also includes questions concerning quantity and frequency of all commonly used drugs including cannabis, alcohol and cigarettes. Participants are asked to respond to questions with the past year as frame of reference. Additionally, at each follow up interview, the CIDI-Substance Abuse Module (CIDI-SAM) (Cottler et al., 1989) which includes questions concerning lifetime use was also administered.

2.3.3. Calculation of lifetime exposure—For the present analyses, information concerning lifetime cannabis, alcohol and cigarette use was derived from the K-SADS, CIDI, and CIDI-SAM interview data. Exposure was calculated based on reported use across the lifespan starting from the first visit until the last visit prior to the scan. From these data participants were classified into a User or Non-User group for whole brain analyses, and further classified into three groups for more refined SPSS analyses aimed at assessing the effects of level of exposure. For these analyses, a median split for users was determined and a three group contrast constructed for each substance (0 for Non-Users, 1 for Below Median Users and 2 for those in the Above the Median group). Each group had a similar number of follow-up visits with mean and standard deviations of 7.69 ± 3.1 for Non-Users; 8.76 ± 2.2 for Below Median Users and 6.71 ± 3.1 for the Above the Median Group, a nonsignificant difference ($F=2.11$, $df=2$, 85 , $p=NS$).

2.4. Assessment of socioeconomic status (SES)

All participants were evaluated for socioeconomic status based on the education and occupation of the parents at the time of the first childhood assessment. SES scores were based on the Hollingshead Four Factor Index of Social Status (Hollingshead, 1975) which

uses a numeric average of the family occupation and education scores. The index groups scores into five major strata: unskilled laborers (8–19), semi-skilled workers (20–29), skilled craftsmen, clerical and sales workers (30–39), minor professional and technical or owner of medium size business (40–54), major business and professional (55–66).

2.5. Assessment of prenatal use of substances

At the time the offspring were entered into the follow-up study, the first of several follow-up visits to our laboratory, the mother was administered a structured interview (Drinking and Drug Use During Pregnancy) concerning her alcohol, cigarette, and other drug use during each of her pregnancies so that the quantity and frequency of these substances could be determined and used as control variables.

2.6. Informed consent and safety monitoring

All participants signed informed consent documents after having the study explained to them. All were screened to insure absence of ferromagnetic metal in or on their body. All female subjects were screened for absence of pregnancy using Icon® 25 hCG pregnancy kits. A neuroradiologist reviewed any scan considered suspicious for abnormality.

2.7. Image acquisition

Each participant received a single MRI scan performed at the University of Pittsburgh Medical Center (UPMC) Magnetic Resonance Research Center (MRRC) using a 3T head-only Siemens Trio scanner (Siemens Medical Systems, Erlangen, Germany) equipped with a fast gradient system for echoplanar imaging. A standard radiofrequency head coil was used with foam padding to restrict head motion. A 7-min 3D T1-weighted Magnetization Prepared Rapid Gradient Echo Imaging (MPRAGE) sequence (TR=2300 ms, TE=2.98 ms, FA=9°, field of view FOV=240 mm, acquisition matrix=240×256, in-plane resolution 1.0×1.0 mm², yielding 160 transversal slices with a thickness of 1.2 mm) was used to acquire a high-resolution anatomical scan for VBM analysis.

2.8. Preprocessing

Data preprocessing was performed using Statistical Parametric Mapping software SPM8 (<http://www.fil.ion.ucl.ac.uk/spm/>) and the VBM8 toolbox (<http://dbm.neuro.uni-jena.de/vbm/>). Data for 19 subjects failed to pass the quality standards and were excluded from further analysis. The structural images were bias corrected, segmented into gray, white, and cerebrospinal fluid, affine registration, normalization, and modulation performed. VBM8 uses a maximum posteriori method to segment tissue types, with the segmented images normalized to Montreal Neurological Institute (MNI) space using nonlinear DARTEL normalization. The voxel size was resliced to 1×1×1 mm³ for all images.

Manual quality checks of the VBM8 modulated gray matter (GM) images were performed to determine if any structural abnormalities were present followed by a check of all of the images for homogeneity and absence of outliers. Gray matter images with the poorest covariance were removed from the analysis. These quality controlled modulated images were then smoothed using a 12-mm full-width at half-maximum (FWHM) isotropic Gaussian kernel.

SPM statistical analyses of the gray matter images were performed using a two sample *t*-test with contrasts defined as cannabis Non-users greater than Users. The resulting maps were thresholded at $p < 0.005$ with a cluster size of 50 voxels. Family-wise error (F_{we}) rates were calculated to adjust for multiple testing. Additionally, regional volumes were calculated using the MarsBaR ROI toolbox (<http://marsbar.sourceforge.net>) to compare the GM volume of the specified regions of interests (ROIs) which were further compared in SPSS (version 20). These ROIs calculated for each participant enabled us to perform analyses in SPSS to follow up on areas revealed in the whole brain analyses using multiple covariates of interest.

2.9. DNA isolation and genotyping

Genomic DNA extracted from whole blood or from EBV transformation and cryopreservation was amplified using PCR incorporating a biotinylated primer. Genotyping was completed on a Biotage PSQ 96MA Pyrosequencer (Biotage AB, Uppsala, Sweden). The biotinylated single strand was isolated from the double strand PCR products and sequenced using the complementary sequencing primer. Quality control of SNP genotyping included ongoing monitoring using Qiagen software. SNPs were chosen giving preference to those in exons, minor allele frequency ($MAF > 0.12$), and greater frequency of published reports concerning the particular SNP. Four CNR1 SNPs were selected for genotyping. Of the five exonic SNPs available, two met these criteria. Ensembl GRCh38 (Release 84) localizes these two SNPs to Exon 5 (rs806368, rs1049353). Additionally, two intronic SNPs were chosen, one in Intron 3 (rs2023239), and one in Intron 2 (rs6454674) (Fig. 1). Two SNPs (rs806368 and rs6454674) were genotyped using the reverse complement.

2.10. Haplotype inference

Linkage disequilibrium (LD) analysis was performed using HAPLOVIEW version 4.2 (Barrett et al., 2005) calculating D' values pair-wise between the four SNPs (Fig. 2). The offspring haplotypes were estimated using the program PHASE, version 2.1.1 (Stephens et al., 2001; Stephens and Donnelly, 2003).

2.11. Statistical analysis

Whole brain analysis was conducted using SPM8. In order to avoid Type 2 error, all regions showing significant uncorrected p values were tested using a mixed model (SPSS) where fixed effects included level of cannabis exposure (0, 1, 2) with “0” representing no use, “1” representing those below the median for the tested sample, and “2” representing those above the median of the sample. A random effect was included to control for possible influence of including multiple sibs from the same family. To limit multiple testing effects, haplotypes were constructed from the four SNPs. The main effect of cannabis exposure, haplotype variation and their interaction were first tested using all potential confounder effects (SES, sex, lifetime personal exposure to alcohol or cigarettes, prenatal exposure to alcohol and cigarettes). Those effects found to be significant for the haplotype were further tested at the SNP level. The SNP level analysis was performed to provide replication, or lack thereof, for SNPs previously reported to influence cannabis exposure effects such as those found for rs2023239 by Schacht et al. (2012).

3. Results

3.1. Participant characteristics

Analysis was performed for 88 scans (Table 1). The 88 participants had been followed 13.0 ± 4.1 years (median years=14). A total of 54 individuals reported no use of marijuana in either the KSADS or CIDI interviews. The CIDI uses a <5 times in lifetime as a criterion for skip out of further questions regarding quantity and frequency of any drug use. Because this level of use over the number of years followed indicates a very low level of use, these subjects data were included in the nonuser group. The remaining 34 users were divided into two groups based on a median split of their lifetime frequency of use reported from multiple waves of data collected during the course of a longitudinal follow-up. An approximately equal number were from families with multiplex alcohol dependence background or families with minimal familial alcohol dependence (low-risk controls). There were 43 high-risk offspring (Mean age 27.4 ± 3.6 years) and 45 low-risk offspring (Mean age 24.5 ± 4.1 years).

Individuals in the cannabis Below the Median group with lesser frequency of use also had a lower percentage of cases meeting criteria for cannabis abuse or dependence (11.8%) versus those in the cannabis Above the Median group with cannabis abuse or dependence (82.4%), a statistically significant difference (Table 1). Greater use of cannabis was associated with increasing levels of use of alcohol and cigarettes which was also statistically significant (Table 1).

3.2. Whole brain analysis

The whole brain analysis conducted in SPM8 using age, sex and familial risk status as covariates revealed four regions in which Non-Users of cannabis had larger volumes than did Regular Users with uncorrected p values <0.001 (Table 2). These included the lingual gyrus, anterior cingulum (right and left), and the rolandic operculum (right) (Fig. 3). The right anterior cingulum was significant with family-wise error (F_{we}) correction of $p=0.055$. This VBM result became more significant ($p=0.001$) as the confounding influence of the participant's cigarette smoking and alcohol use were controlled along with any effects of prenatal use of substances by their mothers (Table 3). First, all smokers were removed from the data set and the effects of familial risk, age, sex, and alcohol use were incorporated in the analysis. Although this reduced the sample size to 63 scans, the p value improved to 0.013. Because the longitudinal data base includes information on prenatal alcohol exposure in 57 of these cases, the effect of this exposure could be evaluated. Analysis using this additional covariate improved the p value to 0.001. Fig. 4 illustrates Non-user greater than User variation in these 57 cases. Because of the potential for prenatal cigarette use to have an effect on gray matter, this covariate was added for 54 cases that had information for this variable along with information on their personal use of alcohol and cigarettes, demographic data and familial risk background. The resulting p value for this sample was $p=0.001$.

3.3. Mixed model analyses

The SPM8 whole brain analysis guided further analyses in SPSS (version 20) using each participant's total voxels by region. Results for these analyses are summarized in Table 2. For these analyses, masks from the WFU Pick Atlas were used to define regions for analysis.

In order to insure that significant confounding effects were not missed due to statistical limitations of SPM8, SPSS mixed model analyses were performed. These analyses incorporated a family identification variable as a random effect to control for family relatedness as some of the participants had siblings in the study. Additionally, because one goal of the study was to determine if CNR1 variation might influence the impact of cannabis exposure on brain volumes, SPSS mixed model analyses were better suited for testing these effects. Accordingly, the mixed model analyses were performed with the main effects of Lifetime Frequency group (No Use, Less than the Median, and Greater than the Median Use), haplotype variation, and their interaction tested in a model that included lifetime quantity of alcohol use, and lifetime quantity of cigarette use, SES, sex, prenatal exposure to cigarettes and alcohol. The main effects of cannabis exposure, controlling for these confounder effects were then tested with haplotype variation to determine the main effect of haplotype and their interaction with cannabis exposure level. Each haplotype was tested under a dominant model for haplotype variation using the presence of any 1-1, 1-2, or 2-1, where 1 represents the major allele and 2 the minor allele, and under an additive model using the number of 1-1, 1-2, and 2-1 counts.

3.4. Haplotypes

SNPs rs806368 and rs1049353 were in high LD ($D' = 0.95$), and rs2023239 and rs645674 were also in high LD ($D' = 0.93$) with other pairs having lower LD ($D' = 0.08-0.16$). Marker allele frequencies in our sample were tested for departures from Hardy-Weinberg equilibrium (HWE) using the allele frequency option in MENDEL version 14.4 (Lange et al., 2005, 2013) which estimates population allele frequencies allowing for sample comparisons. Files needed for MENDEL were generated using the program Mega2 (Mukhopadhyay et al., 2005). None of the four SNPs analyzed were found to have p values below the Bonferroni adjusted threshold (0.0125) that would indicate departure from HWE. Imaging results were analyzed for each of the two SNP haplotypes.

3.5. Haplotype analysis

A summary of results for each region found to be significant in the SPM8 analysis and the influence of each haplotype on these regions may be seen in Table 4. All analyses were first run with the full complement of covariates and then with nonsignificant covariates removed. Results appearing in Table 4 represent the best final model.

3.5.1. Anterior cingulum right—Analysis of the 1, 2 haplotype consisting of the rs806368 and rs1049353 SNPs (Fig. 2), lifetime cannabis exposure, and their interaction revealed a significant effect of haplotype of the “any 12” (any TA) variation ($F=8.96$, $df=1$, 63.00 , $p=0.004$), and a significant interaction with cannabis exposure ($F=7.04$, $df=2$, 63.00 , $p=0.002$) (Table 4). Testing under an additive model also revealed a significant interaction ($F=4.87$, $df=3$, 61.00 , $p=0.004$) (Table 4). Changes volume in association with group membership (0=Non-User, 1=User with < Median Use, and 2=User with > Median Use) along with variation in the 1, 2 haplotype seen in Fig. 5 represents a 17.6% reduction in volume for those in the group with use greater than the median with the “any TA” variation in the rs806368 – rs1049353 haplotype.

Significant haplotype effects were followed up with tests for the effects of single SNPs to provide comparison with previous publications in which single SNPs were investigated. Further analysis of the rs1049353 SNP with cannabis use showed a main effect of cannabis exposure ($F=3.34$, $df=2, 72$, $p=0.041$), a main effect of the SNP, rs1049353 ($F=4.21$, $df=1, 72$, $p=0.044$), and an interaction effect such that the presence of any minor allele (A nucleotide) resulted in a significant interaction with level of cannabis use ($F=5.91$, $df=2, 72$, $p=0.004$). A test for the additive effect of the A nucleotide revealed similar effects with the interaction of cannabis use and number of A alleles also significant ($F=4.23$, $df=3, 70$, $p=0.008$).

Analysis of the rs806368 SNP, under a dominant model in which the presence of any minor allele was tested, revealed a significant effect of cannabis (0, 1, 2) ($F=3.81$, $df=2, 72$, $p=0.027$) but a non significant interaction with the presence of the minor allele (C nucleotide).

All tests of the effect of the 3, 4 haplotype consisting of the rs2023239 and rs6454674 SNPs failed to reveal a significant effect of this haplotype on Right Anterior Cingulum volume alone, or in combination with level of cannabis exposure. Further analysis of the SNPs comprising the haplotype showed no significant effects of rs2023239, though the main effect of cannabis exposure was significant ($F=3.19$, $df=2, 72$, $p=0.047$). In contrast, analysis of rs6454674 under both a dominant and additive model showed a significant interaction between level of cannabis use and presence of the minor allele (G nucleotide). For the dominant model, the interaction effect was significant ($F=5.73$, $df=2, 72$, $p=0.005$). For the additive model in which the number of G alleles were analyzed, a significant interaction was also present ($F=3.48$, $df=4, 69$, $p=0.012$).

3.5.2. Anterior cingulum left—Analysis of the two haplotypes did not reveal any statistically significant main effects or interaction with cannabis exposure.

3.5.3. Operculum (Right)—The 3, 4 haplotype analysis revealed significant main effects for cannabis exposure ($F=4.97$, $df=2, 64.86$, $p=0.010$), haplotype variation (any TG) ($F=4.17$, $df=1, 67.12$, $p=0.045$), and their interaction ($F=3.58$, $df=2, 65.85$, $p=0.033$). Significant results were not found for the 1, 2 haplotype.

3.5.4. Lingual gyrus—In an analysis of the effects of cannabis exposure, haplotype variation (presence of a TT), and their interaction, only the main effects of cannabis exposure was seen ($F=3.39$, $df=2, 61.29$, $p=0.040$). Analysis of cannabis exposure, the presence of a TG, and their interaction revealed only an effect of cannabis use ($F=3.15$, $df=2, 59.88$, $p=0.050$). (Table 4).

4. Discussion

The goal of this study was to determine to what extent cannabis use influences regional brain changes. An unbiased plan was first used to determine which regions were most likely to be affected. This whole brain analysis guided the second stage analysis in which regions revealed in the whole brain analysis were further tested using a large array of covariates.

These analyses provided support for results obtained from the whole brain analysis, and revealed interactions between cannabis exposure and CNR1 genotypic variation. Importantly, personal use of cigarettes and alcohol, SES, or maternal prenatal use of substance were included in our analyses so that the impact of the level of cannabis use in association with CNR1 variation on brain volumes could be adequately assessed. The whole brain analysis had revealed reductions in both hemispheres for the anterior cingulum and the operculum (right) in association with cannabis use. Moreover, reduction of the anterior cingulum (right) was moderated by variation in a haplotype that included two SNPs rs1049353 and rs806368 within exon 5, a coding region of the CNR1 gene. These SNPs are 3' UTR variants that can be expected to contain regulatory regions that post-transcriptionally influence gene expression. The effect of cannabis exposure in the presence of altered gene expression of the CB1 receptors may provide an explanation for why reduction of volume in the highest consumers of cannabis are seen if these individuals carry the TA haplotype. Because the anterior cingulum is involved in reward processing, interactions between cannabis use and CNR1 variation that result in structural abnormalities may have important behavioral implications.

Animal studies have documented the neurotoxic effects of cannabis by showing that exposure to delta 9-Tetra-hydrocannabinol (THC), the main psychoactive component of cannabis results in neuronal loss. Early studies showed that chronic exposure to THC results in hippocampal tissue loss (Scallet et al., 1987; Landfield et al., 1988), with further evidence provided by studies in cultured neurons showing apoptosis when exposed to THC (Chan et al., 1998; Downer et al., 2001) and to the synthetic cannabinoid WIN 55,212-2 (Lawston et al., 2000).

Unlike the animal literature, reports concerning the question of possible neurotoxicity in humans are often complicated by differences in study design, extent of cannabis use, personal exposure to alcohol, drugs, and cigarettes along with possible prenatal exposures which are frequently unknown. Four reviews of structural and functional effects of cannabis use have been reported (Martín-Santos et al., 2010; Lorenzetti et al., 2010; Rapp et al., 2012; Batalla et al., 2013). In one of these, 43 studies were reviewed covering both adolescent and adult samples providing evidence of morphological brain alterations (Batalla et al., 2013). Although some inconsistencies were noted, it was concluded that observed differences appear to cluster in the medial temporal, frontal cortex, and cerebellum.

Certainly there are studies showing an absence of significant regional differences between users and non-users of cannabis. However, these negative results may have been due to insufficient power to detect differences with small samples (Block et al., 2000; Jager et al., 2007; DeLisi et al., 2006; Gruber and Yurgelun-Todd, 2005; Medina et al., 2007; Medina et al., 2010). More recent morphological studies with larger samples have found reductions in volume in the anterior and posterior cingulum in patients with heavy use of cannabis and psychosis (Rapp et al., 2013), in the amygdala and hippocampus (Schacht et al., 2012; Cousijn et al., 2012; Gilman et al., 2014; Lorenzetti et al., 2015; Yücel et al., 2008; Pagliaccio et al., 2015), and in the nucleus accumbens (Gilman et al., 2014), along with the medial temporal cortex, temporal pole, parhippocampal gyrus, insula, and orbitofrontal cortex (Battistella et al., 2014; Filbey et al., 2014). Also, functional imaging effects have

been reported for regular marijuana users with enhanced cue-elicited craving in the anterior cingulate gyrus, orbitofrontal cortex, and inferior frontal gyrus seen in association with variation in CNR1 SNP rs2023239 (Filbey et al., 2010).

Inconsistencies in the literature may also be due to an absence of information regarding CNR1 variation. The extent to which cannabis use is associated with structural brain changes appears to be influenced by CNR1 variation as seen in the present study and by others (Schacht et al., 2012). These investigators showed that hippocampal and amygdalar volumes were reduced in heavy cannabis users in comparison to matched controls. They found that the presence of the G allele of the CNR1 intronic SNP rs2023239 in heavy users was associated with lower bilateral hippocampal volume than in controls. Variation in CNR1 SNPs and presence of cannabis dependence (Hopfer et al., 2006; Hartman et al., 2009) and other substance use phenotypes including alcohol and drug dependence (Zuo et al., 2007) have frequently been reported, though these studies did not investigate brain morphology.

Although many studies suggest that cannabis use is associated with regional loss of brain volume, some have questioned whether this reduction is due to cannabis exposure or due to the contaminating influence of other exposures. Recently, Weiland et al. (2015) have questioned whether cannabis is the causative agent in volumetric changes seen in cannabis users noting that the effect of cannabis use is abolished when statistical control of alcohol use is included. The present results are noteworthy because they demonstrate for the first time that reduction in volume in the right anterior cingulate is associated with cannabis use when controlling for lifetime personal use of alcohol and cigarettes. Moreover, prenatal exposure effects were also controlled and the association of reduction in anterior cingulate volume (right) remained. Additionally, the present findings are significant in showing a gene by environment effect for volume reduction in the right anterior cingulate with the effects of cannabis exposure dependent on CNR1 rs806368 and rs1049353 haplotype variation. We are most confident of our results concerning the anterior cingulate because this region met family-wise error correction estimates for significance using whole brain VBM that was confirmed using a mixed model analyses in SPSS that allowed for entry of multiple covariates including sibling effects. However, our results for operculum (right), lingual gyrus, and amygdala also show evidence of cannabis induced reduction of volume when appropriate covariates are applied.

The significance of our findings for the anterior cingulum from a behavioral perspective lies in the potential for long-term use of cannabis to have deleterious effects on motivation. Although there has been some debate over the function of the anterior cingulate cortex (ACC), whether it is a structure dedicated to motivating behavior or designed for cognitive control and reinforcement learning (Holroyd and Yeung, 2012), it is clear that it is involved in goal directed behavior. Non-human primate studies using both lesions of the ACC and neuronal recording have demonstrated that ACC is critical for learning the value of actions (Kennerley et al., 2006; Amemori et al., 2015). An fMRI study examining the influence of motivation on the control hierarchy in the human frontal cortex found that the right anterior cingulate was one of the regions with the most significant $Z_{\max}$ further supporting the importance of this region in motivation (Bahlmann et al., 2015). If long-term use of cannabis is associated with reduction in volume of the right anterior cingulum, the potential for

changes in motivated behaviors appears to be a possibility. The notion that heavy cannabis use leads to an “amotivational syndrome” has a long history (McGlothlin and West, 1968; Smith, 1968; Carlin and Post, 1974). The contention that such a syndrome exists was based on early observations that middle class individuals previously characterized by a high level of achievement-oriented behavior became excessively relaxed individuals without future goals following heavy use of cannabis. Although the existence of this syndrome continues to be debated, the description of such marked changes in behavior in a subset of individuals who regularly use large quantities of cannabis continues to be worthy of study. Neuropathological changes occurring in the right anterior cingulum might provide the neurobiological basis for this syndrome. The present results also suggest that, perhaps, not all individuals are equally vulnerable to such neuropathological alteration, in part due to genetic variation in the CNR1 gene.

There are limitations to the present study that should be mentioned. Because our analysis was based on longitudinal follow up of all available offspring from high and low risk for alcohol dependence families, participants were not selected on the basis of their cannabis use to be among the highest consumers of cannabis. Accordingly, the average number of years of documented use was 4.6 years. Other reports have been based on selected samples of heavy users of cannabis with an average of 10 years of use (Schacht et al., 2012). Additionally, the present sample included participants with a wide range in onset of cannabis use from 13 to 31 years so that some participants were adolescents when they began using while others were young adults. In view of the possible greater vulnerability to the neurotoxic effects of cannabis during adolescence (Jacobus et al., 2009), those participants who began using cannabis in young adulthood may have experienced lesser neurotoxicity.

Although there are limitations in the present study, several positive aspects were present. These include the prospective nature of data collection that made it possible to quantify lifetime exposure to cannabis, alcohol, cigarette, and other drug exposures without relying on lifetime retrospective report. This feature allowed for assessing lifetime exposure up to the time of the scan for cannabis and enabled us to covary lifetime use of alcohol and cigarettes which are commonly used along with cannabis. Additionally, the study combined an unbiased whole brain approach with directed statistical analyses based on guidance from our whole brain analysis. Finally, the present study provides replication of previous reports of an interaction between CNR1 variation and cannabis exposure in reduction of selected brain volumes. Our results showing an interaction between CNR1, cannabis use, and volume of the anterior cingulum may provide evidence that a subset of individuals may be especially vulnerable to the effects of cannabis use. Moreover, those with neuropathological changes in the anterior cingulum, a region having a clear role in motivated behavior, may have greater likelihood of behavioral change consistent with what was first described as the amotivational syndrome. Also, anterior cingulate deficits have been reported in association with cannabis use in first-episode schizophrenia, a condition frequently characterized by apathy (Szeszko et al., 2007).

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References

- Agarwal A, Lynskey MT. The genetic epidemiology of cannabis use, abuse, and dependence. *Addiction*. 2006; 104:518–532.
- Amemori K-I, Amemori S, Graybiel AM. Motivation and affective judgments differentially recruit neurons in the primate dorsolateral prefrontal and anterior cingulate cortex. *J. Neurosci*. 2015; 35:1939–1953. [PubMed: 25653353]
- Bahlmann J, Aarts E, D'Esposito MD. Influence of motivation on control hierarchy in the human frontal cortex. *J. Neurosci*. 2015; 35:3207–3217. [PubMed: 25698755]
- Bangalore SS, Prasad KM, Montrose DM, Goradia DD, Diwadkar VA, Keshavan MS. Cannabis use and brain structural alterations in first episode schizophrenia – a region of interest, voxel based morphometric study. *Schizophr. Res*. 2008; 99:1–6. [PubMed: 18248793]
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*. 2005; 21:263–265. [PubMed: 15297300]
- Batalla A, Bhattacharyya S, Yücel M, Fusar-Poli P, Crippa JA, Nogué S, Torrens M, Pujol J, Farré M, Martin-Santos R. Structural and functional imaging studies in chronic cannabis users: a systematic review of adolescent and adult findings. *PLoS One*. 2013; 8:e55821. [PubMed: 23390554]
- Battistella G, Fornari E, Annoni JM, Chtioui H, Dao K, Fabritius M, Favrat B, Mall JF, Maeder P, Giroud C. Long-term effects of cannabis on brain structure. *Neuropsychopharmacology*. 2014; 39:2041–2048. [PubMed: 24633558]
- Block RI, O'Leary DS, Ehrhardt JC, Augustinack JC, Ghoneim MM, Arndt S, Hall JA. Effects of frequent marijuana use on brain tissue volume and composition. *Neuroreport*. 2000; 11:491–496. [PubMed: 10718301]
- Carlin AS, Post RD. Drug use and achievement. *Int. J. Addict*. 1974; 9:401–410. [PubMed: 4443136]
- Chambers W, Puig-Antich J, Hirsch M, Paez P, Ambrosini P, Tabrizi M, Davies M. The assessment of affective disorders in children and adolescents by semistructured interview: test–retest reliability of the schedule for affective disorders and schizophrenia for school-age children, present episode version. *Arch. Gen. Psychiatry*. 1985; 42:696–702. [PubMed: 4015311]
- Chan GC, Hinds TR, Impey S, Storm DR. Hippocampal neurotoxicity of Delta9-tetrahydrocannabinol. *J. Neurosci*. 1998; 18:5322–5332. [PubMed: 9651215]
- Childers SR, Breivogel CS. Cannabis and endogenous cannabinoid systems. *Drug Alcohol Depend*. 1998; 51:173–187. [PubMed: 9716939]
- Cottler LB, Robins LN, Helzer JE. The reliability of the CIDI-SAM: a comprehensive substance abuse interview. *Br. J. Addict*. 1989; 84:801–814. [PubMed: 2758153]
- Cousijn J, Wiers RW, Ridderinkhof KR, van den Brink W, Veltman DJ, Goudriaan AE. Grey matter alterations associated with cannabis use: results of a VBM study in heavy cannabis users and healthy controls. *Neuroimage*. 2012; 59:3845–3851. [PubMed: 21982932]
- DeLisi LE, Bertisch HC, Szulc KU, Majcher M, Brown K, Bappal A, Ardekani BA. A preliminary DTI study showing no brain structural change associated with adolescent cannabis use. *Harm Reduct. J*. 2006; 3:17. [PubMed: 16684342]
- Downer E, Boland B, Fogarty M, Campbell V. Delta 9-tetrahydrocannabinol induces the apoptotic pathway in cultured cortical neurones via activation of the CB1 receptor. *Neuroreport*. 2001; 12:3973–3978. [PubMed: 11742222]

- Eggan SM, Lewis DA. Immunocytochemical distribution of the cannabinoid CB1 receptor in the primate neocortex: a regional and laminar analysis. *Cereb. Cortex.* 2007; 17:175–191. [PubMed: 16467563]
- Filbey FM, Schacht JP, Myers US, Chavez RS, Hutchison KE. Individual and additive effects of the CNR1 and FAAH genes on brain response to marijuana cues. *Neuropsychopharmacology.* 2010; 35:967–975. [PubMed: 20010552]
- Filbey FM, Aslan S, Calhoun VD, Spence JS, Damaraju E, Caprihan A, Segall J. Long-term effects of marijuana use on the brain. *Proc. Natl. Acad. Sci. USA.* 2014; 111:16913–16918. [PubMed: 25385625]
- Gilman JM, Kuster JK, Lee S, Lee MJ, Kim BW, Makris N, van der Kouwe A, Blood AJ, Brieter HC. Cannabis use is quantitatively associated with nucleus accumbens and amygdala abnormalities in young adult recreational users. *J. Neurosci.* 2014; 34:5529–5538. [PubMed: 24741043]
- Gruber SA, Yurgelun-Todd DA. Neuroimaging of marijuana smokers during inhibitory processing: a pilot investigation. *Brain Res. Cogn. Brain Res.* 2005; 23:107–118. [PubMed: 15795138]
- Hall W, Solowij N. Adverse effects of cannabis. *Lancet.* 1998; 352:1611–1616. [PubMed: 9843121]
- Hartman CA, Hopfer CJ, Haberstick B, Rhee SH, Crowley TJ, Corley RP, Hewitt JK, Ehringer M. The association between cannabinoid receptor 1 gene (CNR1) and cannabis dependence symptoms in adolescents and young adults. *Drug Alcohol Depend.* 2009; 104:11–16. [PubMed: 19443135]
- Herkenham M, Lynn AB, Johnson MR, Melvin LS, deCosta BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *J. Neurosci.* 1991; 11:563–583. [PubMed: 1992016]
- Hill SY, Tessner KD, McDermott MD. Psychopathology in offspring from families of alcohol dependent female probands: a prospective study. *J. Psychiatr. Res.* 2011; 45:285–294. [PubMed: 20801463]
- Ho B-C, Wassnik TH, Ziebell S, Andreasen NC. Cannabinoid receptor 1 gene polymorphisms and marijuana misuse interactions on white matter and cognitive deficits in schizophrenia. *Schizophr. Res.* 2011; 128:66–75. [PubMed: 21420833]
- Hollingshead, AB. Four Factor Index of Social Status. Department of Sociology, Yale University: New Haven, CT: 1975.
- Holroyd CB, Yeung N. Motivation of extended behaviors by anterior cingulate cortex. *Trends Cogn. Sci.* 2012; 16:122–128. [PubMed: 22226543]
- Hopfer CJ, Young SE, Purcell S, Crowley TJ, Stallings MC, Corley R, Rhee SH, Smolen A, Krauter K, Hewitt JK, Ehringer MA. Cannabis receptor haplotype protective against developing cannabis dependence symptoms in adolescents. *Am. Med. Genet.* 2006; 141B:895–901.
- Jacobus J, Bava S, Cohen-Zion M, Mahmood O, Tapert SF. Functional consequences of marijuana use in adolescents. *Pharmacol. Biochem. Behav.* 2009; 92:559–565. [PubMed: 19348837]
- Jager G, Van Hell HH, De Win MM, Kahn RS, Van Den Brink W, Van Ree JM, Ramsey NF. Effects of frequent cannabis use on hippocampal activity during an associative memory task. *Eur. Neuropsychopharmacol.* 2007; 17:289–297. [PubMed: 17137758]
- Janca A, Robins LN, Cottler LB, Early TS. Clinical observation of assessment using the Composite International Diagnostic Interview (CIDI). An analysis of the CIDI field trials B wave II at the St. Louis site. *Br. J. Psychiatry.* 1992; 160:815–818. [PubMed: 1617365]
- Johnston, LD.; O'Malley, PM.; Bachman, JG.; Schulenberg, JE. Monitoring the Future: National results on drug use, 1975–2013 – Overview of Key Findings on Adolescent Drug Use. Institute for Social Research, University of Michigan; Ann Arbor: 2013. 2014. <http://www.monitoringthefuture.org/pubs/monographs/mtf-overview2013.pdf>
- Kennerley SW, Walton ME, Behrens TE, Buckley MJ, Rushworth MFS. Optimal decision making and the anterior cingulate cortex. *Nat. Neurosci.* 2006; 9:940–947. [PubMed: 16783368]
- Landfield PW, Cadwallader LB, Vinsant S. Quantitative changes in hippocampal structure following long-term exposure to delta 9-tetrahydrocannabinol: possible mediation by glucocorticoid systems. *Brain Res.* 1988; 443:47–62. [PubMed: 2834017]
- Lange K, Sinsheimer JS, Sobel EM. Association testing with Mendel. *Genet. Epidemiol.* 2005; 29:36–50. [PubMed: 15834862]

- Lange K, Papp JC, Sinsheimer JS, Sripracha R, Zhou R, Sobel EM. The Swiss army knife of genetic analysis programs. *Bioinformatics*. 2013; 29:1568–1570. [PubMed: 23610370]
- Lawston J, Borella A, Robinson JK, Whitaker-Azmitia PM. Changes in hippocampal morphology following chronic treatment with the synthetic cannabinoid WIN 55,212-2. *Brain Res*. 2000; 877:407–410. [PubMed: 10986361]
- Lorenzetti V, Lubman DI, Whittle S, Solowij N, Yücel M. Structural MRI findings in long-term cannabis users: what do we know? *Subst. Use Misuse*. 2010; 45:1787–1808. [PubMed: 20590400]
- Lorenzetti V, Solowij N, Whittle S, Fornito A, Lubman DI, Pantelis C, Yücel M. Gross morphological brain changes with chronic, heavy cannabis use. *Br. J. Psychiatry*. 2015; 206:77–78. [PubMed: 25431432]
- Martín-Santos R, Fagundo AB, Crippa JA, Atakan Z, Bhattacharyya S, Allen P, Fusar-Poli P, Borgwardt S, Seal M, Busatto GF, McGuire P. Neuroimaging in cannabis use: a systematic review of the literature. *Psychol. Med*. 2010; 40:383–398. [PubMed: 19627647]
- Matochik JA, Eldreth DA, Cadet JL, Bolla KI. Altered brain tissue composition in heavy marijuana users. *Drug Alcohol Depend*. 2005; 77:23–30. [PubMed: 15607838]
- McGlothlin WH, West LJ. The marihuana problem: an overview. *Am. J. Psychiatry*. 1968; 125:370–378.
- Medina KL, Nagel BJ, Park A, McQueeney T, Tapert SF. Depressive symptoms in adolescents: associations with white matter volume and marijuana use. *J. Child. Psychol. Psychiatry*. 2007; 48:592–600. [PubMed: 17537075]
- Medina KL, Nagel BJ, Tapert SF. Abnormal cerebellar morphometry in abstinent adolescent marijuana users. *Psychiatry Res*. 2010; 182:152–159. [PubMed: 20413277]
- Mukhopadhyay N, Almasy L, Schroeder M, Mulvihill WP, Weeks DE. Mega2: data-handling for facilitating genetic linkage and association analyses. *Bioinformatics*. 2005; 21:2556–2557. [PubMed: 15746282]
- Murray RM, Morrison PD, Henquet C, Di Forti M. Cannabis, the mind and society: the hash realities. *Nat. Rev. Neurosci*. 2007; 8:885–895. [PubMed: 17925811]
- Pagliaccio, D.; Barch, DM.; Bogdan, R.; Wood, PK.; Lynskey, MT.; Heath, AC.; Agrawal, A. Shared predisposition in the association between cannabis use and subcortical brain structure.. *JAMA Psychiatry*. 2015. <http://dx.doi.org/10.1001/jamapsychiatry.2015.1054>, Published online August 26, 2015
- Rapp C, Bugra H, Riecher-Rössler A, Tamagni C, Borgwardt S. Effects of cannabis use on human brain structure in psychosis: a systematic review combining in vivo structural neuroimaging and post mortem studies. *Curr. Pharm. Des*. 2012; 18:5070–5080. [PubMed: 22716152]
- Rapp C, Walter A, Studerus E, Bugra H, Tamagni C, Röthlisberger M, Borgwardt S, Aston J, Riecher-Rössler A. Cannabis use and brain structural alterations of the cingulate cortex in early psychosis. *Psychiatry Res*. 2013; 214:102–108. [PubMed: 24054726]
- Robins N, Helzer JE, Croughan J, Ratcliff KS. National Institute of Mental Health diagnostic interview schedule. *Arch. Gen. Psychiatry*. 1981; 38:381–389. [PubMed: 6260053]
- Scallet AC, Uemura E, Andrews A, Ali SF, McMillan DE, Paule MG, Brown RM, Slikker W Jr. Morphometric studies of the rat hippocampus following chronic delta-9-tetrahydrocannabinol (THC). *Brain Res*. 1987; 436:193–198. [PubMed: 2825925]
- Schacht JP, Hutchison KE, Filbey FM. Associations between cannabinoid receptor-1 (CNR1) variation and hippocampus and amygdala volumes in heavy cannabis users. *Neuropsychopharmacology*. 2012; 37:2368–2376. [PubMed: 22669173]
- Smith DE. Acute and chronic toxicity in marihuana. *J. Psychedelic Drugs*. 1968; 2:37–47.
- Stephens M, Donnelly P. A comparison of Bayesian methods for haplotype reconstruction. *Am. J. Hum. Genet*. 2003; 73:1162–1169. [PubMed: 14574645]
- Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet*. 2001; 68:978–989. [PubMed: 11254454]
- Substance Abuse and Mental Health Services Administration. Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No. (SMA) 14-4863. Substance Abuse and Mental Health Services Administration; Rockville,

MD: 2014. < <http://www.samhsa.gov/data/sites/default/files/NSDUHresultsPDFHTML2013/Web/NSDUHresults2013.pdf> >

- Szeszko PR, Robinson DG, Sevy S, Kumra S, Rupp CI, Betensky JD, Lencz T, Ashtari M, Kane JM, Malhotra AK, Gunduz-Bruce H, Napolitano B, Bilder RM. Anterior cingulate grey-matter deficits and cannabis use in first-episode schizophrenia. *Br. J. Psychiatry.* 2007; 190:230–236. [PubMed: 17329743]
- Weiland BJ, Thayer RE, Depue BE, Sabbineni A, Bryan AD, Hutchison KE. Daily marijuana use is not associated with brain morphometric measures in adolescents or adults. *J. Neurosci.* 2015; 35:1505–1512. [PubMed: 25632127]
- Yücel M, Solowij N, Respondek C, Whittle S, Fornito A, Pantelis C, Lubman DI. Regional brain abnormalities associated with long-term heavy cannabis use. *Arch. Gen. Psychiatry.* 2008; 65:694–701. [PubMed: 18519827]
- Zuo L, Kranzler HR, Luo X, Covault J, Gelernter J. CNR1 variation modulates risk for drug and alcohol dependence. *Biol. Psychiatry.* 2007; 62:616–626. [PubMed: 17509535]

Fig. 1.

The CNR1 gene localized to 6q14-q15 is shown with genotyped markers. The position of each single nucleotide polymorphism (SNP) is shown in relation to each exon (represented by a numbered shaded box). The minor allele frequency for these SNPs listed in Ensembl GRCh38 (Release 84) are: 0.270 (rs806368), 0.129 (rs1049353), 0.178 (rs2023239), and 0.314 (rs6454674). The brain cannabinoid receptor (CB1) is encoded by the CNR1 gene.

Fig. 2. Linkage disequilibrium (LD) analysis was performed using HAPLOVIEW version 4.2 (Barrett et al., 2005) calculating D' values pair-wise between the four SNPs.

Fig. 3.

A whole brain analysis conducted in SPM8 using age, sex and familial risk status as covariates revealed four regions in which Non-Users of cannabis had larger volumes than did Regular Users with a threshold of 50 voxels with a p value < 0.005 . These included the lingual gyrus, anterior cingulum (right and left), and the rolandic operculum (right). The right anterior cingulum was significant with family-wise error (F_{we}) correction of $p = 0.055$.

Fig. 4.
The longitudinal data base included information on prenatal alcohol exposure in 57 cases. Analysis using this additional covariate improved the p value to 0.001. Figure illustrates Non-user greater than User variation in these 57 cases.

Fig. 5.

Figure illustrates the effect of cannabis exposure moderated by CNR1 variation. Analysis of the rs806368 and rs1049353 haplotype showed a significant interaction between the presence of any TA within this haplotype and cannabis exposure ($F=7.08$, $df=2$, 64.7 , $p=0.002$). A linear model in which the number of TA variants was tested was also significant ($F=4.97$, $df=2$, 64.24 , $p=0.004$). The rs806368 and rs1049353 are 3' UTR variants that have been localized to exon 5 of the CNR1 gene.

Table 1

Participant characteristics by cannabis frequency group, cannabis abuse and cannabis dependence.

	All participants	Cannabis – no use (N=54)	Cannabis < median (N=17)	Cannabis > median (N=17)	Significance
Age	25.9 ± 4.09	25.4 ± 4.3	27.2 ± 4.3	26.4 ± 2.8	NS
Male (%)	44.3	48.1	70.6	47.1	NS
African-American/Caucasian (%)	10.0	8.0	4.0	4.0	NS
Years of education	14.8 ± 2.0	14.9 ± 1.9	15.1 ± 2.3	14.1 ± 4.1	NS
SES					
(First at median age 11 years)	44.0 ± 13.1	48.4 ± 11.0	39.5 ± 13.1	34.7 ± 13.7	F=8.14, df=2,72, p=0.001; F=5.18, df=2,85, p=0.008
(At scan: median age 25.5 years)	44.3 ± 9.1	46.4 ± 8.3	43.5 ± 9.2	38.7 ± 9.4	
Peabody Picture Vocabulary IQ					F=3.41, df=2,85, p=0.038; F=6.26, df=2,85, p=0.003
(First at median age 12 years)	105.5 ± 14.7	108.0 ± 13.7	105.6 ± 15.3	97.6 ± 15.4	
(At Scan: median age 25 years)	101.4 ± 11.8	104.5 ± 10.2	98.9 ± 14.0	94.0 ± 10.9	
Years of cannabis use	4.6 ± 3.4 ^a	0	3.00 ± 2.9	6.3 ± 3.1	
(Mean/Median/Range)	0		2 years	6 years	F=75.59, df=2,85, p<0.001
	0–13 years		1–13 years	1–12 years	
Age of onset cannabis use	18.3 ± 4.8 ^a		18.1 ± 4.4	18.5	
(Mean/Median/Range)	17	0	17	17	NS
	13–31		14–30	13–31	
Lifetime cannabis frequency	502.8 ± 643.2 ^a	0	20.5 ± 18.3	985.2 ± 598.8	F=97.58, df=2,85, p<0.001
(Mean/Median/Range)	130.3		15.0	988.0	
	4–2060.5		4–58.5	202–2060.5	
Lifetime cigarette use	2214.9 ± 19,711.2 ^b	1774.3 ± 7574.7	9167.9 ± 16,770.9	17,756.2 ± 21,036.3	
(Mean/Median/Range)	9855.0	0	0	8577.5	F=10.11, df=2,85, p<0.001
	2190–55,297.5	0–53,837.5	0–55,297.5	0–54,385	
Lifetime alcohol use	2195.7 ± 2489.9 ^c	909.5 ± 1276.1	2521.2 ± 2107.3	4147.4 ± 3595.4	
(Mean/Median/Range)	1677.0	234.0	2593.5	3705.0	F=16.94, df=2,85, p<0.001
	26–11,947.0	0–6435.0	0–8944.0	0–11,947.0	
CIDI Cannabis Abuse (All)	8/88 (9.1%)				
CIDI Cannabis Abuse (By group)		0	1/17 (5.9%)	7/17 (41.2%)	$\chi^2=22.79$, df=2, p<0.001
CIDI Cannabis Dependence (All)	11/88(12.5%)				
CIDI Cannabis Dependence (By group)		0	2/17(11.8%)	9/17 (52.9%)	$\chi^2=33.14$, df=2, p<0.001
CIDI Cannabis Abuse or Dependence (By group)	16/88 (18.2%)	0	2/17 (11.8%)	14/17 (82.4%)	$\chi^2=59.53$, df=2, p<0.001

Tests of significance are based on one-way ANOVAs for continuous variables and Chi Square values for frequency measures.

^aNumber of days used cannabis up to time of scan – average based on cannabis users only (N=34).^bNumber of cigarettes used up to time of scan – values are based on smokers only (N=25).

^cNumber of alcohol drinks – values are based on alcohol users only (N=74).

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Table 2

Significant clusters in whole brain analysis non-users > users.

		<u>Voxel statistics</u>			<u>Cluster statistics</u>				
		<u>MNI coordinate (mm)</u>							
Localization	Brodmann area	x	y	z	Peak Z	<i>p</i> (uncorr)	Size (<i>k</i>)	<i>p</i> (uncorr)	<i>p</i> (FWE)
Lingual gyrus	BA 18	2	-78	-12	3.75	<0.001	142	0.030	NS
Anterior cingulum left	BA 9	-3	42	19	3.56	<0.001	276	0.004	NS
Anterior cingulum right	BA 32	5	32	24	3.81	<0.001	423	0.001	0.055
Rolandic operculum right	BA 13, 41, 42	53	-28	22	3.86	<0.001	321	0.002	NS

MNI=Montreal Neurological Institute.

FWE=Family Wise Error.

Voxel and Cluster Statistics: Main effects of cannabis use versus non-use was analyzed controlling for age, risk, and sex.

Mixed Model analysis performed in SPSS included sex, socioeconomic status (SES), lifetime quantity of alcohol use, lifetime quantity of cigarette use, prenatal use of alcohol, cigarettes, and other drugs by the mothers.

Table 3

Right anterior cingulum non-users > users.

Full sample (N=88)						
Covariates=Familial risk, scan age, sex						
Voxel statistics				Cluster statistics		
MNI coordinate (mm)						
x	y	z	Peak Z	p _{uncorr}	Size (k)	p(FWE)
5	32	24	3.81	<0.001	423	0.055
Non-smokers of cigarettes sample (N=63) ^a						
Covariates=Familial risk, scan age, sex, personal use of alcohol						
2	33	21	3.78	<0.001	536	0.013
Non-smokers of cigarettes sample (N=57) ^b						
Covariates=Familial risk, scan age, sex, personal use of alcohol, prenatal alcohol exposure						
9	33	16	3.86	<0.001	774	0.001
Non-smokers of cigarettes sample (N=54) ^c						
Covariates=Familial risk, scan age, sex, personal use of alcohol, prenatal alcohol exposure, prenatal drug exposure, prenatal cigarette exposure						
11	32	16	4.65	<0.001	1347	<0.001

MNI=Montreal Neurological Institute. FWE=Family Wise Error.

Voxel statistics refer to peak effects within a cluster. Cluster statistics reflects a greater number of contiguous voxels exceeding $p < 0.005$ (uncorrected) than expected by chance, after controlling for whole brain FWE.^a15 smokers were removed from the analysis.^bData concerning prenatal alcohol exposure was not available for 6 cases.^cData concerning prenatal exposure to drugs and cigarettes were not available for an additional 3 cases.

Table 4

Haplotype, cannabis exposure, and their interaction effects on the anterior cingulum (right and left), operculum (right) and lingual gyrus (total volume).

	CANNABIS	CNR1	CNR1 × CANNABIS	
HAPLOTYPE 12:				
CGTA OR TGTA (ANY TA) ^a	*	0.004	0.002 ^b	RT ANT CINGULUM
TATA ^a	*	0.028	0.004 ^b	RT ANT CINGULUM
HAPLOTYPE 34				
CTTG OR TTTG (ANY TG) ^a	0.010	0.045	0.033	OPERCULUM RT
TTTT ^a	0.040	*	*	LINGUAL GYRUS
CTTG OR TTTG (ANY TG) ^a	0.050	*	*	LINGUAL GYRUS
HAPLOTYPE 12= rs806368 and rs1049353 ^c				
HAPLOTYPE 34= rs2023239 and rs6454674 ^d				

Analyses were corrected for family ID, sex, SES, Lifetime Use of Alcohol, Lifetime Use of Cigarettes, Prenatal Exposure to Alcohol, Prenatal Exposure to Cigarettes. Non-significant covariates were removed and analysis rerun for the final model presented here.

^aThe final model included total amount of prenatal alcohol exposure as it was significant in the full model that included all covariates.

^bUsing a Bonferroni correction and the conventional level of significance of 0.05, each of the four regions would need to have a p-value of <0.004. The interaction of cannabis use and CNR1 variation for the Right Anterior Cingulum are less than the required corrected value.

^cThe frequency of haplotype 12 was 0.52, 0.26, and 0.22 for the 11, 12, and 21 haplotypes, respectively.

^dThe frequency of haplotype 34 was 0.50, 0.27 and 0.24 for the 11, 12, and 21 haplotypes, respectively.