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Externalizing Behaviors are associated with SNPs in the *CHRNA5/CHRNA3/CHRNA4* gene cluster

Sarah H. Stephens^{1,4}, Nicole R. Hoft⁴, Isabel R. Schlaepfer⁵, Susan E. Young⁴, Robin C. Corley⁴, Matthew B. McQueen^{1,4}, Christian Hopfer⁶, Thomas Crowley⁶, Michael Stallings^{2,3,4}, John Hewitt^{2,4}, and Marissa A. Ehringer^{1,4,*}

¹Departments of Integrative Physiology, University of Colorado Boulder

²Departments of Psychology, University of Colorado Boulder

³Departments of Neuroscience, University of Colorado Boulder

⁴Institute for Behavioral Genetics, University of Colorado Boulder

⁵Division of Endocrinology, Metabolism, and Diabetes, University of Colorado Denver

⁶Departments of Psychiatry, University of Colorado Denver

Abstract

There is strong evidence for shared genetic factors contributing to childhood externalizing disorders and substance abuse. Externalizing disorders often precede early substance experimentation, leading to the idea that individuals inherit a genetic vulnerability to generalized disinhibitory psychopathology. Genetic variation in the *CHRNA5/CHRNA3/CHRNA4* gene cluster has been associated with early substance experimentation, nicotine dependence, and other drug behaviors. This study examines whether the *CHRNA5/CHRNA3/CHRNA4* locus is correlated also with externalizing behaviors in three independent longitudinally assessed adolescent samples. We developed a common externalizing behavior phenotype from the available measures in the three samples, and tested for association with 10 SNPs in the gene cluster. Significant results were detected in two of the samples, including rs8040868, which remained significant after controlling for smoking quantity. These results expand on previous work focused mainly on drug behaviors, and support the hypothesis that variation in the *CHRNA5/CHRNA3/CHRNA4* locus is associated with early externalizing behaviors.

Keywords

nicotinic receptor genes; externalizing behaviors; association study; disinhibition; drug behaviors

INTRODUCTION

Early experimentation with substances and substance use disorders (SUDs) in adolescence are highly co-morbid with a spectrum of childhood behavioral disorders including Attention Deficit Hyperactivity Disorder (ADHD), Conduct Disorder (CD), Oppositional Deviant Disorder (ODD), delinquency, and criminal behavior (McGue et al. 2001; Han et al. 1999; Armstrong and Costello 2002; Young et al. 2000; Krueger et al. 2002). In fact, these types of childhood disorders, along with personality traits such as high levels of novelty seeking

*Corresponding Author: Marissa Ehringer, Ph.D., Department of Integrative Physiology, Institute for Behavioral Genetics, University of Colorado Boulder, 447 UCB, Boulder, CO 80309-0447, 303-492-1463, 303-492-8063 (FAX), Marissa.Ehringer@colorado.edu.

(NS) and impulsivity often precede early substance experimentation (Young et al. 2000; Flory et al. 2003; Weinberg et al. 1998; McGue and Iacono 2008; Tarter et al. 2003). There is evidence from multiple independent studies that individuals inherit a genetic vulnerability to generalized disinhibitory psychopathology including SUD, CD, ADHD, and novelty seeking personality traits (Young et al. 2000; Iacono et al. 2008; Prescott and Kendler 1999). These disinhibitory behaviors are distinguished by the inability to inhibit socially undesirable or restricted actions despite consequences to the individual (legal and psychologically) and/or their family members (Young et al. 2000; Iacono et al. 2008). While the genetic vulnerability to early-onset disinhibitory psychopathology may lead to social, familial and legal consequences as an adolescent, it is also associated with increased risk of problems in adulthood. These may include mental-health problems, difficulty in personal and family relationships, substance dependence, criminality, and economic failure (Taylor et al. 2000; Moffitt et al. 2002; McGue and Iacono 2005). Therefore, knowledge of the genetic factors which manifest as adolescent problems of disinhibition will further our understanding of a developmental trajectory that includes substance use and lifelong psychopathology.

Family, twin, and adoption studies strongly implicate genetic factors underlying childhood behavioral disorders such as ADHD and other externalizing problems (Hicks et al. 2004; Biederman and Faraone 2005; Faraone et al. 2005; Thapar et al. 2005). Heritability estimates range from 0.75 to 0.91. Furthermore, multivariate behavior genetics models have provided evidence that common genetic factors contribute to ADHD and other externalizing disorders such as ODD and CD (Young et al. 2000; Young et al. 2006; Anney et al. 2008; Thapar et al. 2001; Vierikko et al. 2004; Nadder et al. 2002).

Likewise, the influence of genetic factors on substance abuse is well established in the literature (Uhl et al. 2008; Uhl 2004). Identification of genetic vulnerabilities to substance use and abuse during adolescence has been of particular importance since adolescence is the time where individuals experiment with substances and establish patterns of behavior that often persist into adulthood. Early initiation of substances is a well-established risk factor for the succession to abuse and dependence (McGue et al. 2001; Prescott and Kendler 1999; Bucholz et al. 2000; Chen and Anthony 2003; Grant and Dawson 1998; DeWit et al. 2000; Hingson et al. 2006; Breslau et al. 1993). As is the case with childhood behavioral disorders, there is strong evidence for common genetic factors contributing to the abuse of multiple substances (Gruza and Bierut 2006; Stinson et al. 2005; True et al. 1999; Hopfer et al. 2001). Twin studies have uncovered a significant genetic correlation between multiple substances of abuse including tobacco, alcohol, opioids, cocaine, cannabis where inherited factors explained between 22–45% of the variation in drug abuse and/or dependence (Hopfer et al. 2001; Pickens et al. 1995; Tsuang et al. 1996; Swan et al. 1996; van den Bree et al. 1998; Merikangas et al. 1998; Bierut et al. 1998; Grove et al. 1990; Kendler et al. 2003). In agreement with reports on the etiology of the comorbidity of substance use across multiple substances in adults, studies in adolescents also suggest a common underlying genetic component across substances (Han et al. 1999; Young et al. 2006; Koopmans et al. 1999; Stallings et al. 2003).

In recent years, much attention has been given to the *CHRNA5/CHRNA3/CHRNA4* gene cluster on chromosome 15 for numerous replicated associations with multiple substances of abuse. This cluster of neuronal nicotinic acetylcholine receptor (nAChR) subunit coding genes on 15q21 was first implicated with nicotine dependence in multiple independent studies (Rose 2007; Greenbaum and Lerer 2009). Additionally, this region contains a functionally important non-synonymous risk variant in the *CHRNA5* gene (Bierut et al., 2008). The *CHRNA5/CHRNA3/CHRNA4* gene cluster has been associated with nicotine dependence (ND) and smoking behaviors in seven genome-wide association studies

(GWAS) (Berrettini et al. 2008; Bierut et al. 2007; Caporaso et al. 2009; Furberg et al. 2010; Liu et al. 2010; Thorgeirsson et al. 2008; Thorgeirsson et al. 2010). In candidate gene association studies, SNPs in the *CHRNA5/CHRNA3/CHRNA4* cluster have been associated with ND (Bierut et al. 2007; Li et al. 2009; Saccone et al. 2009; Baker et al. 2009; Weiss et al. 2008; Saccone et al. 2009; Chen et al. 2009; Spitz et al. 2008; Bierut et al. 2008; Sherva et al. 2010) as well as smoking behaviors including ND severity (Greenbaum et al. 2006), heavy smoking (Stevens et al. 2008), nicotine intake (via serum cotinine levels) (Keskitalo et al. 2009), 'pleasurable buzz' during early experimentation (Sherva et al. 2008), and early age of initiation (Schlaepfer et al. 2008). In addition, SNPs in these genes have been associated with increased risk for lung cancer (Spitz et al. 2008; Sasaki et al. 2009; Le Marchand et al. 2008; Amos et al. 2008; Hung et al. 2008; Schwartz et al. 2009). Furthermore, the *CHRNA5/CHRNA3/CHRNA4* locus has been associated with alcohol dependence (Sherva et al. 2010), level of response to alcohol (Joslyn et al. 2008), and alcohol age of initiation (Schlaepfer et al. 2008). Chen *et al.* (2009) reported an association of SNPs in *CHRNA5* and *CHRNA3* with both ND and symptoms of alcohol use and dependence. Additionally, two SNPs in *CHRNA5* (rs16969968 and rs684513) are associated with cocaine dependence (Bierut et al. 2008; Sherva et al. 2010). Interestingly, the non-synonymous SNP in *CHRNA5*, rs16969968 (G/A), was associated with cocaine dependence (Gruzca et al. 2008), but the risk allele (A) for ND (Saccone et al. 2009; Chen et al. 2009; Bierut et al. 2008; Stevens et al. 2008; Saccone et al. 2007), smoking behaviors (Weiss et al. 2008; Sherva et al. 2008), lung cancer (Hung et al. 2008), and alcohol dependence (Chen et al. 2009) was protective for cocaine dependence. Functional studies with SNP rs16969968 (G/A) found that the A allele decreased response to a nicotine agonist (Bierut et al. 2008).

For the current study, we sought to examine whether variation in the *CHRNA5/CHRNA3/CHRNA4* gene cluster also predicts disinhibitory and/or externalizing behaviors in three longitudinally assessed adolescent/young adult samples. We utilized SNPs which have been shown by our group to be associated with both early tobacco and alcohol initiation in young adults (Schlaepfer et al. 2008) and those shown to be associated with nicotine and/or other substance abuse/dependence.

METHODS

This study has examined three samples referred to as the Add Health sample, the CADD sample, and the NYF-FS sample, each described in more detail below. Two of these samples have been analyzed previously by our group for alcohol and tobacco behaviors (Schlaepfer et al. 2008; Hoft et al. 2009). Only three SNPs in the *CHRNA5/CHRNA3/CHRNA4* gene cluster had been analyzed previously for association with CPD in the Add Health sample as part of a meta-analysis (Saccone et al. 2010).

Our aim was to harmonize the phenotype across all three samples in order to test the central hypothesis that these genes are associated with externalizing behaviors in adolescents, thus putting them at increased risk for substance abuse. For the CADD sample, we included six lifetime DSM-IV CD symptoms corresponding to those items constructed from the available criteria-relevant questions in the other two data sets. Our efforts resulted in six "externalizing" behaviors (initiating fights, using a weapon, stealing with confrontation, vandalism, breaking and entering, and stealing without confrontation). The Add Health sample contains a number of items that approximate the externalizing behaviors for CD, and questions were categorized to reflect those criteria. Some of these behaviors were unable to be included in the Add Health sample because criteria-relevant questions were not asked or were not consistently asked across waves. These same externalizing behaviors were constructed for the NYS-FS family-based sample using the available data from waves II, III, and VI in order to capture age groups that mirrored those from the CADD and Add Health

samples. Supplementary Tables IA and IB show questions included in the constructed externalizing behaviors for the Add Health (**IA**) and NYS-FS (**IB**) samples categorized by wave of assessment. In addition, it was important to control for smoking quantity as this measure has shown the most robust associations with SNPs in the *CHRNA5/CHRNA3/CHRNA4* (Furberg et al. 2010; Liu et al. 2010; Thorgeirsson et al. 2010)

Samples

The National Longitudinal Study of Adolescent Health (Add Health)—The National Longitudinal Study of Adolescent Health (Add Health) (<http://www.cpc.unc.edu/projects/addhealth/projects/addhealth>). This study sample consists of adolescents in grades 7–12 in the United States during the 1994–1995 school year. Individuals were systematically selected from 80 high schools and 52 middle schools in order to ensure the sample was representative of United States schools with respect to region, urbanicity, school size, school type, and ethnicity. The Add Health cohort has been followed into young adulthood with four in-home interviews (corresponding to waves I, II, III, IV). The most recent interview occurred in 2008, when the sample was aged 24–32. Wave IV data were not included in the analysis in order to focus on behavioral problems in adolescence. Survey data assessed respondents' social, economic, psychological and physical well-being with contextual data on family, neighborhood, community, school, friendships, peer groups, and romantic relationships (<http://www.cpc.unc.edu/projects/addhealth/codebooks>). The genetic pairs sample includes pairs of individuals with varying genetic similarity including monozygotic twins, dizygotic twins, full siblings, half siblings, and unrelated siblings who were raised in the same household. During wave III interviews, a sample of N = 2,611 respondents was selected from the sibling pairs data and asked to submit samples of their DNA for genotyping (Harris et al. 2006). Of the 2611 genetic pairs sample, there were 1476 (48% male) European-Americans for whom both genotypic and phenotypic data were available.

Externalizing behaviors were derived from the “Delinquency and Violence” questions asked during waves I, II, and III. We created six items (initiating fights, using a weapon, stealing with confrontation, vandalism, breaking and entering, and stealing without confrontation) from criteria-relevant questions from waves I–III for a total of 18 items (Supplementary Table IA). Questionnaires were scored for the past year as 0 = never engaged in the behavior, 1 = engaged in the behavior one or two times, 2 = engaged in the behavior 3 or 4 times, and 3 = engaged in the behavior five or more times. CPD was assessed for the period of heaviest smoking (wave III questionnaire) and from current use for waves I, II, and III; the maximum of these values was used to define the CPD trait for analyses.

Center for the Genetics of Antisocial Drug Dependence—We evaluated a subset of unrelated European-American subjects from the family, twin, and adoption studies of the Colorado Center for the Genetics of Antisocial Drug Dependence (CADD) <http://ibgwww.colorado.edu/cadd>. This ongoing, multi-component, collaborative study includes over 5000 individuals from both clinical and community populations. Clinical probands were recruited from three treatment facilities in the Denver metropolitan area. Clinical subjects comprised 16% of the sample analyzed for this study. The probands were 13–19 years of age at time of assessment and were drawn from individuals with consecutive admissions to the treatment facilities between February 1993 and June 2001. The community-based sample included monozygotic and dizygotic twins and their non-twin siblings drawn from the Colorado Twin Registry. Additional community samples were drawn from two other Colorado community-based family samples: The Colorado Adoption Project (CAP) (DeFries et al. 1994) and the Colorado Adolescent Substance Abuse (ASA) family study (Stallings et al. 2003; Stallings et al. 2005). DNA samples were collected on a voluntary basis by buccal swabs. All study participants were given cognitive, psychiatric,

and socio-demographic assessments that included both structured diagnostic interviews as well as self-reported questionnaires. A description of the study was presented to all subjects, who signed written informed assent (minors) or consent (adults) to participate. Of the 5000 youths surveyed for the CADD, we selected those assessed between ages 17 and 21 (mean age 18 ± 1.50) for inclusion in this study. Details regarding selection criteria are described in Schlaepfer et al. 2008. Our subset included 730 (54% male) European-American subjects.

In order to harmonize the phenotype, we utilized only DSM-IV (lifetime) conduct disorder symptoms that mirrored those items constructed from the criteria-relevant questions in the Add Health data set (initiating fights, using a weapon, stealing with confrontation, vandalism, breaking and entering, and stealing without confrontation). Individuals were given a score of 0 (never engaged in that behavior) or 1 (engaged in the behavior). Cigarettes per day was defined as the maximum number reported across waves I and II.

National Youth Survey – Family Study—The NYS-FS is a nationally representative probability-sample of households containing subjects aged 11–17 in 1976 and living in the United States in 1977 (<http://www.colorado.edu/ibs/NYSFS>). At that time 1,725 youths, as well as one of their parents, were interviewed. Five waves of data were collected from this sample at yearly intervals between 1977 and 1981. A sixth wave was collected in 1984, and a seventh in 1987 (Elliott et al. 1989). In 2002, a follow-up interview was conducted (35–44 years) during which behavioral data and DNA samples were collected on a voluntary basis by buccal swabs. A total of 1071 individuals both agreed to follow-up interviews and provided DNA samples. This subsample appears to retain its representativeness of the United States population (Hoft et al. 2009). Data from waves II, III, and VI (adolescence) were included in the analysis to align with those ages collected in the Add Health study. Of the 1071 DNAs available, we used the 860 (48% male) European-American subjects.

In accordance with the Add Health data, the same six externalizing behavioral items were constructed from criteria-relevant questions (Sakai et al. 2010) (Supplementary Table IB). Questionnaires were scored for the past year as 1 = engaged in the behavior once a month, 2 = engaged in the behavior every 2–3 weeks, 3 = engaged in the behavior once a week, 4 = engaged in the behavior 2–3 times a week, 5 = engaged in the behavior once a day, and 6 = engaged in the behavior 2–3 times per day. Cigarettes per day was defined as the maximum number reported across the public waves (II, III, VI).

SNP Selection and Genotyping

Candidate polymorphisms for the *CHRNA5/CHRNA3/CHRNA4* gene cluster had been previously genotyped in the CADD and NYS-FS samples (Schlaepfer et al. 2008) with the exception of rs16969968 (*CHRNA5*), which was added for this study since it is a non-synonymous SNP with evidence for functional relevance (Bierut et al. 2008). Briefly, the candidate SNPs were identified with the SNPbrowser Software version 3.5 from Applied Biosystems (<http://www.appliedbiosystems.com>) and the public database, dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>). Genomic DNA was preamplified with the method of Zheng et al. (Anchordoquy et al. 2003; Zheng et al. 2001). SNP genotyping was performed by TaqMan® assays for allelic discrimination according to the manufacturer's instructions (Applied Biosystems, Foster City, California). Polymerase Chain Reaction (PCR) reactions with specific primers and probes was accomplished with the Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter Inc, Brea, California) and the Dual 384-Well GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California). The 7900 Real-Time PCR System (Applied Biosystems, Foster City, California) was utilized to analyze PCR-amplified 384-well plates.

Between 100 and 400 samples were genotyped at least twice to confirm a minimum of 99% concordance between replicate reactions. Genotype clusters were auto-called by the Applied Biosystems TaqMan® Genotyper software (Applied Biosystems, Foster City, California) and then visually examined by two independent lab personnel to make calls. Genotype calls from each person were compared and if different, the data were not used.

Statistical Methods

Data descriptive—Pairwise linkage disequilibrium (LD) (r^2) and conformity to Hardy-Weinberg equilibrium (HWE) was assessed using Haploview (Barrett et al. 2005). For the CADD sample, all individuals were included to test for conformity to HWE, in the family-based samples (NYS-FS, Add Health) one individual per family was randomly selected.

Analysis of the Add Health and NYS-FS samples—Since the Add Health and NYS-FS samples contain family members, family-based association tests (FBAT) were carried out as part of the PBAT software package (Laird and Lange 2006; Lange et al. 2004). This allowed us to capitalize on the FBAT principal component (FBAT-PC) option (Lange et al. 2004) which is designed to maximize the genetic information when multiple phenotypes are analyzed. In addition, FBAT can be applied to non-normal data as it makes no distributional assumptions about the trait. In our study, there were three waves of data including six items each for Add Health and NYS-FS (18 total items for each sample). Raw questionnaire scores for the Add Health (0–3) and NYS-FS (1–6) were entered into FBAT-PC as a quantitative variable. For each SNP tested, FBAT-PC maximizes the heritability of the principal component using the between family information. Family-based tests of association are run using the constructed phenotype using the within family information. Cigarettes per day (CPD), gender, age, and age² were included as covariates. For our secondary analysis with individual externalizing behaviors and SNPs found to be significant with FBAT-PC, we utilized FBAT-GEE as part of the PBAT software package (Lange et al. 2003). While FBAT-GEE was designed to test multiple phenotypes at once, selection of a single phenotype in PBAT gives the test results for a standard quantitative FBAT (Laird et al. 2000).

Analysis of the CADD sample—The six (lifetime) externalizing behaviors were summed so each individual scored between zero and six. In the CADD community sample, symptom counts were expressed as z-score deviations from the expected values based on the regression of symptom counts on sex, age, and age². For the clinical sample the symptom counts were expressed as z-score deviations from expected values based on the same community sample prediction equation. The resulting z-scores were log transformed to be used in the analysis. The correlation between the raw symptom count and the final variable was 0.84 ($p < 0.0001$). This phenotype was analyzed with standard linear regression in SAS version 9.2 software (SAS Institute Inc.: Cary, NC, 2004). Cigarettes per day was included as a covariate in the model.

Significance levels for all analyses were defined at $P = 0.007$ based on the SNPSpD program. This method corrects for multiple markers using the spectral decomposition of matrices of pairwise linkage disequilibrium between markers (Nyholt 2004). This significance level does not account for multiple phenotypes. FBAT-PC generated one composite phenotype for each of the family-based data sets (Add Health and NYS-FS), and one phenotype was tested in the CADD sample, so a total of three tests were performed.

RESULTS

Linkage disequilibrium (LD) and Hardy Weinberg Conformance

Table I presents descriptive genetic information generated by Haploview including SNP location, gene, minor allele frequency (MAF), call rates, and HWE p-values. Using a p-value threshold for HWE of $p < 0.001$ (based on Haploview (Barrett et al. 2005) and PLINK (Purcell et al. 2009)), two SNPs in the Add Health subjects (rs11637630 and rs1316971) and two SNPs in the NYS-FS sample (rs8040868 and rs8023462) did not meet Hardy-Weinberg expectations. Because of the geometry of the Taqman VIC/FAM (dye) cluster plots, conservative genotype calls lead to a larger no-call rate for heterozygote genotypes than homozygote genotypes. In the CADD sample, all markers were in Hardy-Weinberg Equilibrium (HWE). Supplementary Figures 1A, 1B and 1C show the LD plots generated by Haploview for the ten SNP markers in each sample population. SNPs rs8023462 and rs1948 were highly correlated in all three samples (Add Health $r^2 = 0.80$, CADD $r^2 = 0.91$, NYS-FS $r^2 = 0.74$).

SNPs in the *CHRNA5/CHRNA3/CHRNA4* are associated with externalizing behaviors

Table II summarizes all results. Columns one and two show each SNP and gene. Columns three shows p-values for the Add Health data. Similarly, columns four through five show p-values for the CADD and NYS-FS samples. Results generated by FBAT-PC for the Add Health family data revealed that SNP rs8040868 ($p=0.04$) was significantly associated with the constructed externalizing behavior phenotype. In addition, SNPs rs684513 ($p=0.06$) and rs11634351 ($p=0.054$) showed a trend towards significance. In support of results in the Add Health sample, linear regression with the normalized six item score in the CADD sample showed that SNP rs8040868 was associated ($p=0.007$) with an elevated number of externalizing behaviors while SNPs rs8023462 ($p=0.018$) and rs1948 ($p=0.033$) reached nominal significance. Finally, in the NYS-FS sample, SNP rs680244 was associated with the constructed phenotype ($p=0.049$) while SNP rs16969968 showed a trend towards significance ($p=0.05$). After correction for multiple comparisons, the p-value for rs8040868 passed the threshold for significance in the CADD sample.

Looking at the data for the putatively associated SNPs in more detail, Figures 1 and 2 show mean item symptom count by genotype. For Add Health subjects, questionnaires were scored as 0 = never engaged in the behavior, 1 = engaged in the behavior one or two times, 2 = engaged in the behavior 3 or 4 times, and 3 = engaged in the behavior five or more times. However, to determine associated genotypes we recoded individuals as either 0 (never engaged in that behavior) or 1 (engaged in the behavior). Figure 1 (rs8040868) shows that the minor allele is associated with a slightly elevated mean symptom count in the Add Health sample. In the CADD sample, Figure 2 (rs8040868), also shows that the minor allele for rs8040868 appears to be associated with an elevated mean symptom count in an additive fashion.

Assessing the phenotype—Although we were limited to six symptoms of conduct disorder in the NYS-FS and Add Health samples, all 15 symptoms were available in the CADD sample. In order to assess the strength of our 6 symptom phenotype we first looked at the correlation between the 15 symptom phenotype and the 6 symptom phenotype in the CADD sample. The two phenotypes were highly correlated ($r^2 = 0.91$) indicating the 6 symptom phenotype is representative of Conduct Disorder in the CADD sample. Additionally, we repeated the primary analysis utilizing all 15 symptoms (z-scored in a similar manner) in the CADD sample with linear regression. P-values were very similar to the initial analysis with significance for the same SNPs in the full CADD sample (rs8040868 $p=0.02$, rs8023462 $p=0.009$, rs1948 $p=0.016$). This supports our decision to focus on the six

items that were commonly assessed across all three samples, and suggests our findings may be clinically relevant in the context of diagnosed CD.

Deconstructing composite phenotypes—The FBAT-PC (Lange et al. 2004) approach allowed us to take advantage of our longitudinal data and multiple phenotypes. A useful feature of FBAT-PC is that one can determine which items are weighted most highly in the derivation of the composite phenotype by examining the β weights. These weights were used to construct the composite phenotype for each SNP and analyze the correlation with each item. Figure 3 shows the correlations between the composite of SNP rs8040868 ($p=0.04$) and the individual item scores for all 18 items in the Add Health sample. For rs8040868, Stealing wave III appears to be primarily driving the association. As a secondary analysis, we examined SNP rs8040868 and item Stealing III. Results of FBAT-GEE (Lange et al. 2003) showed Stealing III was not significantly associated with rs8040868 ($p=0.281$). Taken together, these results illustrate the utility of the principal components approach to allow inclusion of other items that contribute important additional information to the analyses.

Post hoc analysis of cigarettes per day phenotype—All three of these samples were analyzed for association between CPD and three SNPs in this region as part of a meta-analysis (Saccone et al. 2010), and as a post-hoc analysis we examined CPD with all SNPs genotyped. As a summary, in the CADD subjects there was no evidence for association with CPD (Schlaepfer et al. 2008; Saccone et al. 2010), three significant SNPs were significant in the NYS-FS sample (rs16969968, rs680244, rs684513), and three SNPs were significant in Add Health subjects (rs16969968, rs11637630, and rs588765).

DISCUSSION

Results from this study provide the first evidence that variation in the *CHRNA5/A3/B4* gene cluster region is associated with externalizing symptoms in adolescent subjects. The most robust finding in this study is at SNP rs8040868 that replicated in two of the three samples (and passed the threshold for multiple corrections in the CADD sample), and appears to be a statistically independent locus. This report expands upon prior work in two ways. First, these genes have never before been examined in the Add Health sample. Second, by examining “externalizing symptoms” as a phenotype we expand the hypothesis that specific genetic variants in this region predisposes an individual toward general disinhibitory psychopathology, which is likely to put them at greater risk for substance use and dependence (Young et al. 2000; Iacono et al. 2008; Prescott and Kendler 1999). We predicted that variation in this region would also be associated with externalizing behaviors in three longitudinally assessed samples. This hypothesis supports the idea that early disinhibition may contribute to a trajectory leading to substance abuse and dependence and/or lifelong mental health problems (Taylor et al. 2000; Moffitt et al. 2002; McGue and Iacono 2005).

We initiated this analysis to examine whether variations in *CHRNA5/CHRNA3/CHRNA4* cluster were correlated with general disinhibitory behaviors. In order to harmonize the phenotype across our three sample populations (CADD, Add Health, NYS-FS), we focused on six externalizing items included in the DSM-IV conduct disorder symptoms and assessed across all samples. SNPs rs8023462, rs1948, and rs8040868 in the *CHRNA5/CHRNA3/CHRNA4* region had been previously shown to be associated with early initiation of alcohol and nicotine use in the CADD and NYS-FS samples (Schlaepfer et al. 2008). In the present study exonic SNP rs8040868 (*CHRNA3*) was nominally associated with externalizing symptoms in Add Health ($p=0.04$) and statistically significant CADD ($p=0.007$) samples. In addition, SNPs rs8023462 ($p=0.018$) and rs1948 ($p=0.033$) were nominally associated with

externalizing behaviors in the CADD sample. This SNP has also previously been associated with ND (Li et al. 2009) and showed a trend for association with early age of initiation of alcohol and nicotine (Schlaepfer et al. 2008). SNP rs680244 was nominally associated with externalizing symptoms ($p=0.049$) in the NYS-FS sample. Rs680244 has been previously associated with severity of nicotine dependence in a model including the effects of both environmental and physiological measures (Greenbaum et al. 2006).

Results from the current study mapped onto distinct ND association signals in the *CHRNA5/CHRNA3/CHRNA4* gene cluster

At least four distinct ND association signals in the *CHRNA5/CHRNA3/CHRNA4* cluster have been identified (Saccone et al. 2009; Saccone et al. 2010). Table II (column 6) shows results of the current study mapped onto the distinct nicotine dependence association signals in the *CHRNA5/CHRNA3/CHRNA4* gene cluster as described in the these previous reports, which will be described here. Locus 1 is a group of highly correlated SNPs tagged by rs16969968. Although SNP rs16969968 (*CHRNA5*) showed a “trend” toward significance in the NYS-FS sample, it was not significantly associated with externalizing symptoms in the three samples employed for this study. This SNP has been most robustly associated with smoking quantity in recent meta-analyses (Furberg et al. 2010; Liu et al. 2010; Thorgeirsson et al. 2010) but was not associated with smoking initiation (Furberg et al. 2010; Thorgeirsson et al. 2010). Thus, the lack of association between rs16969968 and externalizing behaviors in this study is consistent with the literature examining smoking initiation.

Locus 2 is tagged by rs578776 and associated with ND in European-American sample populations (Saccone et al. 2009; Weiss et al. 2008; Saccone et al. 2009). In the current study, SNP rs11637630 is a correlate of Locus 2 tag SNP rs578776 (Hapmap CEU $r^2 = 0.89$). This SNP was not associated with externalizing symptoms in both the CADD and NYS-FS samples. In the Add Health sample, this SNP did not conform to Hardy-Weinberg proportions and was excluded from analysis.

A third independent locus is associated with ND and alcohol dependence (Wang et al. 2009; Wang et al. 2009). Locus 3 is tagged by rs588765 and is also associated with altered *CHRNA5* mRNA levels in human brain (Wang et al. 2009). According to Hapmap, SNPs rs514743 and rs680244 are correlates of rs588765 (Locus 3) (CEU $r^2 = 0.87$ and $r^2 = 1.0$, respectively) and highly correlated with one another ($r^2 = 0.84$). SNP rs680244 was associated with the externalizing phenotype in the NYS-FS sample ($p=0.049$) but not the Add Health or the CADD sample populations. It is interesting to note that SNPs rs514743 and rs680244 were not well correlated in any of our three sample groups (Add Health $r^2 = 0.65$, CADD $r^2 = 0.40$ and NYS-FS $r^2 = 0.57$). These results suggest that our sample LD structure may be different from the Hapmap database for Locus 3 and that we may have captured this locus with rs680244 (NYS-FS) but not rs514743.

The fourth locus is tagged by non-synonymous SNP rs12914008. This locus is rare (4.5% in European-Americans and 0% in African-Americans) and associated with ND in a European-American sample (Saccone et al. 2009). Neither this SNP, nor any of its correlates were surveyed for the current study.

Study Limitations

Limitations of this study mainly apply to our defined “externalizing behaviors” phenotype. For the CADD sample, behaviors were endorsed with a “yes” or a “no” regarding whether the individual engaged in the behavior. Conversely, in the Add Health and NYS-FS samples, subjects reported frequency of the behavior. Additionally, externalizing behavior “items”

constructed from questionnaires differed slightly in their definitions due to wording of the questions. For example, in the Add Health and CADD questionnaires, the “fighting” item was based on a broad definition of fighting, whereas the NYS questionnaire focused on gang fighting. Small differences in the wording of the questionnaire can potentially cause much larger changes in endorsement rates. These disparities in our definition of the phenotype across samples may help to explain why there is not more cross sample replication of specific SNPs.

In addition, there was limited power to detect an association in the NYS-FS. First, the NYS-FS sample DNA quality is relatively poor, leading to fewer subjects genotyped (an average of 83% compared with CADD at 95% and Add Health at 90%). From there, although the full sample included 865 individuals, the number of informative families available for FBAT-PC was between 57(rs11637630) and 79(rs680244). The Add Health sample was twice this size, corresponding to almost three times as many informative families because of slight differences in family structures (135 informative families for SNP rs1316971 and 229 for rs8040868). Another explanation for these results may be cohort differences the NYS-FS and our other two samples (CADD and Add Health). Although we analyzed NYS-FS data from those waves corresponding to the same age range as the CADD and Add Health data, both the CADD and Add Health individuals were between ~12 and 19 in the 1990s while the NYS-FS subjects were adolescents during the 1970s. This 20-year cohort difference may help to explain these disparate results.

Finally, we initiated these analyses to expand on our previous finding of association with tobacco/alcohol age of initiation (Schlaepfer et al. 2008), and the possibility that this is related to early disinhibitory psychopathology. Although one association remained significant after controlling for the effect of smoking quantity, the most appropriate analysis would investigate externalizing behavior in non-smokers. Unfortunately, there are few individuals who score high for externalizing behavior who do not smoke, and thus the ability to completely disentangle the two phenotypes is limited.

Study assets

A strength of this study is that we developed a uniform phenotype in three separate independently ascertained samples. Additionally, we were able to take advantage of longitudinal data available for two of these samples (Add Health and NYS-FS). Our use of an elegant statistical approach (FBAT-PC) for the family-based data allowed us to utilize longitudinal data and multiple phenotypes that are likely to contribute important additional information to the analyses.

This is the first study to examine externalizing behaviors and SNPs located in the *CHRNA5/CHRNA3/CHRNA4* cluster, which has been associated with nicotine, alcohol, and other substance use behaviors in multiple studies. It is the first examination of these genes in the Add Health study, and expands on previous reports by evaluating a novel early phenotype known to be a precursor to substance use and abuse. In addition, this study provides a detailed analysis of LD patterns in these genes providing additional evidence for up to five independent signals that may be functionally distinct. These results support our hypothesis that variation in the *CHRNA5/CHRNA3/CHRNA4* cluster predicts externalizing behaviors. Additionally, this report suggests that work aimed at teasing apart which of these signals are most closely aligned with specific endophenotypes related to substance abuse is merited. Likewise, it will be critical to expand these human genetic associations using molecular and animal approaches to determine whether certain SNPs lead to differences in gene regulation and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- McGue M, Iacono WG, Legrand LN, Malone S, Elkins I. Origins and consequences of age at first drink I Associations with substance-use disorders, disinhibitory behavior and psychopathology, and P3 amplitude. *Alcohol Clin Exp Res*. 2001; 25(8):1156–1165. [PubMed: 11505047]
- Han C, McGue MK, Iacono WG. Lifetime tobacco, alcohol and other substance use in adolescent Minnesota twins: univariate and multivariate behavioral genetic analyses. *Addiction*. 1999; 94(7): 981–993. [PubMed: 10707437]
- Armstrong TD, Costello EJ. Community studies on adolescent substance use abuse, or dependence and psychiatric comorbidity. *J Consult Clin Psychol*. 2002; 70(6):1224–1239. [PubMed: 12472299]
- Young SE, Stallings MC, Corley RP, Krauter KS, Hewitt JK. Genetic and environmental influences on behavioral disinhibition. *Am J Med Genet*. 2000; 96(5):684–695. [PubMed: 11054778]
- Krueger RF, Hicks BM, Patrick CJ, Carlson SR, Iacono WG, McGue M. Etiologic connections among substance dependence, antisocial behavior, and personality: modeling the externalizing spectrum. *J Abnorm Psychol*. 2002; 111(3):411–424. [PubMed: 12150417]
- Flory K, Milich R, Lynam DR, Leukefeld C, Clayton R. Relation between childhood disruptive behavior disorders and substance use and dependence symptoms in young adulthood: individuals with symptoms of attention-deficit/hyperactivity disorder and conduct disorder are uniquely at risk. *Psychol Addict Behav*. 2003; 17(2):151–158. [PubMed: 12814279]
- Weinberg NZ, Rahdert E, Collier JD, Glantz MD. Adolescent substance abuse: a review of the past 10 years. *Journal of the American Academy of Child and Adolescent Psychiatry*. 1998; 37(3):252–261. [PubMed: 9519629]
- McGue M, Iacono WG. The adolescent origins of substance use disorders. *Int J Methods Psychiatr Res*. 2008; 17(Suppl 1):S30–S38. [PubMed: 18543360]
- Tarter RE, Kirisci L, Mezzich A, Cornelius JR, Pajer K, Vanyukov M, Gardner W, Blackson T, Clark D. Neurobehavioral disinhibition in childhood predicts early age at onset of substance use disorder. *The American journal of psychiatry*. 2003; 160(6):1078–1085. [PubMed: 12777265]
- Iacono WG, Malone SM, McGue M. Behavioral disinhibition and the development of early-onset addiction: common and specific influences. *Annu Rev Clin Psychol*. 2008; 4325:348.
- Prescott CA, Kendler KS. Age at first drink and risk for alcoholism: a noncausal association. *Alcohol Clin Exp Res*. 1999; 23(1):101–107. [PubMed: 10029209]
- Taylor J, Iacono WG, McGue M. Evidence for a genetic etiology of early-onset delinquency. *J Abnorm Psychol*. 2000; 109(4):634–643. [PubMed: 11195987]
- Moffitt TE, Caspi A, Harrington H, Milne BJ. Males on the life-course-persistent and adolescence-limited antisocial pathways: follow-up at age 26 years. *Dev Psychopathol*. 2002; 14(1):179–207. [PubMed: 11893092]
- McGue M, Iacono WG. The association of early adolescent problem behavior with adult psychopathology. *The American journal of psychiatry*. 2005; 162(6):1118–1124. [PubMed: 15930060]

- Hicks BM, Krueger RF, Iacono WG, McGue M, Patrick CJ. Family transmission and heritability of externalizing disorders: a twin-family study. *Archives of general psychiatry*. 2004; 61(9):922–928. [PubMed: 15351771]
- Biederman J, Faraone SV. Attention-deficit hyperactivity disorder. *Lancet*. 2005; 366(9481):237–248. [PubMed: 16023516]
- Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA, Sklar P. Molecular genetics of attention-deficit/hyperactivity disorder. *Biol Psychiatry*. 2005; 57(11):1313–1323. [PubMed: 15950004]
- Thapar A, O'Donovan M, Owen MJ. The genetics of attention deficit hyperactivity disorder. *Human molecular genetics*. 2005; 14(Spec No. 2R):275–282.
- Young SE, Rhee SH, Stallings MC, Corley RP, Hewitt JK. Genetic and environmental vulnerabilities underlying adolescent substance use and problem use: general or specific? *Behav Genet*. 2006; 36(4):603–615. [PubMed: 16619135]
- Anney RJ, Lasky-Su J, O'Dushlaine C, Kenny E, Neale BM, Mulligan A, Franke B, Zhou K, Chen W, Christiansen H, Arias-Vasquez A, Banaschewski T, Buitelaar J, Ebstein R, Miranda A, Mulas F, Oades RD, Roeyers H, Rothenberger A, Sergeant J, Sonuga-Barke E, Steinhausen H, Asherson P, Faraone SV, Gill M. Conduct disorder and ADHD: evaluation of conduct problems as a categorical and quantitative trait in the international multicentre ADHD genetics study. *Am J Med Genet B Neuropsychiatr Genet*. 2008; 147B(8):1369–1378. [PubMed: 18951430]
- Thapar A, Harrington R, McGuffin P. Examining the comorbidity of ADHD-related behaviours and conduct problems using a twin study design. *Br J Psychiatry*. 2001;179224–179229.
- Vierikko E, Pulkkinen L, Kaprio J, Rose RJ. Genetic and environmental influences on the relationship between aggression and hyperactivity-impulsivity as rated by teachers and parents. *Twin Res*. 2004; 7(3):261–274. [PubMed: 15193171]
- Nadder TS, Rutter M, Silberg JL, Maes HH, Eaves LJ. Genetic effects on the variation and covariation of attention deficit-hyperactivity disorder (ADHD) and oppositional-defiant disorder/conduct disorder (Odd/CD) symptomatology across informant and occasion of measurement. *Psychological medicine*. 2002; 32(1):39–53. [PubMed: 11883729]
- Uhl GR, Drgon T, Johnson C, Li CY, Contoreggi C, Hess J, Naiman D, Liu QR. Molecular genetics of addiction and related heritable phenotypes: genome-wide association approaches identify "connectivity constellation" and drug target genes with pleiotropic effects. *Annals of the New York Academy of Sciences*. 2008;1141318–1141381.
- Uhl GR. Molecular genetics of substance abuse vulnerability: remarkable recent convergence of genome scan results. *Annals of the New York Academy of Sciences*. 2004;10251–10213.
- Bucholz KK, Heath AC, Madden PA. Transitions in drinking in adolescent females: evidence from the Missouri adolescent female twin study. *Alcohol Clin Exp Res*. 2000; 24(6):914–923. [PubMed: 10888082]
- Chen CY, Anthony JC. Possible age-associated bias in reporting of clinical features of drug dependence: epidemiological evidence on adolescent-onset marijuana use. *Addiction (Abingdon, England)*. 2003; 98(1):71–82.
- Grant BF, Dawson DA. Age of onset of drug use and its association with DSM-IV drug abuse and dependence: results from the National Longitudinal Alcohol Epidemiologic Survey. *Journal of substance abuse*. 1998; 10(2):163–173. [PubMed: 9854701]
- DeWit DJ, Adlaf EM, Offord DR, Ogborne AC. Age at first alcohol use: a risk factor for the development of alcohol disorders. *Am J Psychiatry*. 2000; 157(5):745–750. [PubMed: 10784467]
- Hingson RW, Heeren T, Winter MR. Age at drinking onset and alcohol dependence: age at onset, duration, and severity. *Arch Pediatr Adolesc Med*. 2006; 160(7):739–746. [PubMed: 16818840]
- Breslau N, Fenn N, Peterson EL. Early smoking initiation and nicotine dependence in a cohort of young adults. *Drug and alcohol dependence*. 1993; 33(2):129–137. [PubMed: 8261877]
- Gruzca RA, Bierut LJ. Co-occurring risk factors for alcohol dependence and habitual smoking: update on findings from the Collaborative Study on the Genetics of Alcoholism. *Alcohol Res Health*. 2006; 29(3):172–178. [PubMed: 17373405]
- Stinson FS, Grant BF, Dawson DA, Ruan WJ, Huang B, Saha T. Comorbidity between DSM-IV alcohol and specific drug use disorders in the United States: results from the National

- Epidemiologic Survey on Alcohol and Related Conditions. Drug and alcohol dependence. 2005; 80(1):105–116. [PubMed: 16157233]
- True WR, Xian H, Scherrer JF, Madden PA, Bucholz KK, Heath AC, Eisen SA, Lyons MJ, Goldberg J, Tsuang M. Common genetic vulnerability for nicotine and alcohol dependence in men. *Arch Gen Psychiatry*. 1999; 56(7):655–661. [PubMed: 10401514]
- Hopfer CJ, Stallings MC, Hewitt JK. Common genetic and environmental vulnerability for alcohol and tobacco use in a volunteer sample of older female twins. *Journal of studies on alcohol*. 2001; 62(6):717–723. [PubMed: 11838905]
- Pickens RW, Svikis DS, McGue M, LaBuda MC. Common genetic mechanisms in alcohol, drug, and mental disorder comorbidity. *Drug and alcohol dependence*. 1995; 39(2):129–138. [PubMed: 8529532]
- Tsuang MT, Lyons MJ, Eisen SA, Goldberg J, True W, Lin N, Meyer JM, Toomey R, Faraone SV, Eaves L. Genetic influences on DSM-III-R drug abuse and dependence: a study of 3,372 twin pairs. *American journal of medical genetics*. 1996; 67(5):473–477. [PubMed: 8886164]
- Swan GE, Carmelli D, Cardon LR. The consumption of tobacco, alcohol, and coffee in Caucasian male twins: a multivariate genetic analysis. *J Subst Abuse*. 1996; 8(1):19–31. [PubMed: 8743766]
- van den Bree MB, Johnson EO, Neale MC, Pickens RW. Genetic and environmental influences on drug use and abuse/dependence in male and female twins. *Drug and alcohol dependence*. 1998; 52(3):231–241. [PubMed: 9839149]
- Merikangas KR, Stolar M, Stevens DE, Goulet J, Preisig MA, Fenton B, Zhang H, O'Malley SS, Rounsaville BJ. Familial transmission of substance use disorders. *Archives of general psychiatry*. 1998; 55(11):973–979. [PubMed: 9819065]
- Bierut LJ, Dinwiddie SH, Begleiter H, Crowe RR, Hesselbrock V, Nurnberger JI Jr, Porjesz B, Schuckit MA, Reich T. Familial transmission of substance dependence: alcohol, marijuana, cocaine, and habitual smoking: a report from the Collaborative Study on the Genetics of Alcoholism. *Arch Gen Psychiatry*. 1998; 55(11):982–988. [PubMed: 9819066]
- Grove WM, Eckert ED, Heston L, Bouchard TJ Jr, Segal N, Lykken DT. Heritability of substance abuse and antisocial behavior: a study of monozygotic twins reared apart. *Biological psychiatry*. 1990; 27(12):1293–1304. [PubMed: 2364118]
- Kendler KS, Jacobson KC, Prescott CA, Neale MC. Specificity of genetic and environmental risk factors for use and abuse/dependence of cannabis, cocaine, hallucinogens, sedatives, stimulants, and opiates in male twins. *The American journal of psychiatry*. 2003; 160(4):687–695. [PubMed: 12668357]
- Koopmans JR, Slutske WS, Heath AC, Neale MC, Boomsma DI. The genetics of smoking initiation and quantity smoked in Dutch adolescent and young adult twins. *Behav Genet*. 1999; 29(6):383–393. [PubMed: 10857244]
- Stallings MC, Corley RP, Hewitt JK, Krauter KS, Lessem JM, Mikulich SK, Rhee SH, Smolen A, Young SE, Crowley TJ. A genome-wide search for quantitative trait loci influencing substance dependence vulnerability in adolescence. *Drug Alcohol Depend*. 2003; 70(3):295–307. [PubMed: 12757967]
- Rose JE. Multiple brain pathways and receptors underlying tobacco addiction. *Biochem Pharmacol*. 2007; 74(8):1263–1270. [PubMed: 17826746]
- Greenbaum L, Lerer B. Differential contribution of genetic variation in multiple brain nicotinic cholinergic receptors to nicotine dependence: recent progress and emerging open questions. *Molecular psychiatry*. 2009; 14(10):912–945. [PubMed: 19564872]
- Berrettini W, Yuan X, Tozzi F, Song K, Francks C, Chilcoat H, Waterworth D, Muglia P, Mooser V. alpha-5/alpha-3 nicotinic receptor subunit alleles increase risk for heavy smoking. *Mol Psychiatry*. 2008; 13(4):368–373. [PubMed: 18227835]
- Bierut LJ, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau OF, Swan GE, Rutter J, Bertelsen S, Fox L, Fugman D, Goate AM, Hinrichs AL, Konvicka K, Martin NG, Montgomery GW, Saccone NL, Saccone SF, Wang JC, Chase GA, Rice JP, Ballinger DG. Novel genes identified in a high-density genome wide association study for nicotine dependence. *Hum Mol Genet*. 2007; 16(1):24–35. [PubMed: 17158188]

- Caporaso N, Gu F, Chatterjee N, Sheng-Chih J, Yu K, Yeager M, Chen C, Jacobs K, Wheeler W, Landi MT, Ziegler RG, Hunter DJ, Chanock S, Hankinson S, Kraft P, Bergen AW. Genome-wide and candidate gene association study of cigarette smoking behaviors. *PloS one*. 2009; 4(2):e4653. [PubMed: 19247474]
- Furberg H, Kim Y, Dackor J, Boerwinkle E, Franceschini N, Ardisino D, Bernardinelli L, Mannucci PM, Mauri F, Merlini PA, Absher D, Assimes TL, Fortmann SP, Iribarren C, Knowles JW, Quertermous T, Ferrucci L, Tanaka T, Bis JC, Furberg CD, Haritunians T, McKnight B, Psaty BM, Taylor KD, Thacker EL, Almgren P, Groop L, Ladenvall C, Boehnke M, Jackson AU, Mohlke KL, Stringham HM, Tuomilehto J, Benjamin EJ, Hwang S, Levy D, Preis SR, Vasani RS, Duan J, Gejman PV, Levinson DF, Sanders AR, Shi J, Lips EH, McKay JD, Agudo A, Barzan L, Bencko V, Benhamou S, Castellsague X, Canova C, Conway DI, Fabianova E, Foretova L, Janout V, Healy CM, Holcatova I, Kjaerheim K, Lagiou P, Lissowska J, Lowry R, Macfarlane TV, Mates D, Richiardi L, Rudnai P, Szeszenia-Dabrowska J, Zaridze D, Znaor A, Lathrop M, Brennan P, Bandinelli S, Frayling TM, Guralnik JM, Milaneschi Y, Perry JRB, Altshuler D, Elosua R, Kathiresan S, Lucas G, Melander O, O'Donnell CJ, Salomaa V, Schwartz SM, Voight BF, Penninx BW, Smit JH, Vogelzangs N, Boomsma DI, de Geus EJC, Vink JM, Willemsen G, Chanock SJ, Gu F, Hankinson SE, Hunter DJ, Hofman A, Tiemeier H, Uitterlinden AG, van Duijn CM, Walter S, Chasman DI, Everett BM, Pare G, Ridker PM, Li MD, Maes HH, Audrain-McGovern J, Posthuma D, Thornton LM, Lerman C, Kaprio J, Rose JE, Ioannidis JPA, Kraft P, Lin D, Sullivan PF. Genome-wide meta-analyses identify multiple loci associated with smoking behavior. *INature genetics*. 2010;441–447.
- Liu JZ, Tozzi F, Waterworth DM, Pillai SG, Muglia P, Middleton L, Berrettini W, Knouff CW, Yuan X, Waeber G, Vollenweider P, Preisig M, Wareham NJ, Zhao JH, Loos RJ, Barroso I, Khaw KT, Grundy S, Barter P, Mahley R, Kesaniemi A, McPherson R, Vincent JB, Strauss J, Kennedy JL, Farmer A, McGuffin P, Day R, Matthews K, Bakke P, Gulsvik A, Lucae S, Ising M, Brueckl T, Horstmann S, Wichmann HE, Rawal R, Dahmen N, Lamina C, Polasek O, Zgaga L, Huffman J, Campbell S, Kooner J, Chambers JC, Burnett MS, Devaney JM, Pichard AD, Kent KM, Satler L, Lindsay JM, Waksman R, Epstein S, Wilson JF, Wild SH, Campbell H, Vitart V, Reilly MP, Li M, Qu L, Wilensky R, Matthai W, Hakonarson HH, Rader DJ, Franke A, Wittig M, Schafer A, Uda M, Terracciano A, Xiao X, Busonero F, Scheet P, Schlessinger D, St Clair D, Rujescu D, Abecasis GR, Grabe HJ, Teumer A, Volzke H, Petersmann A, John U, Rudan I, Hayward C, Wright AF, Kolcic I, Wright BJ, Thompson JR, Balmforth AJ, Hall AS, Samani NJ, Anderson CA, Ahmad T, Mathew CG, Parkes M, Satsangi J, Caulfield M, Munroe PB, Farrall M, Dominiczak A, Worthington J, Thomson W, Eyre S, Barton A, Mooser V, Francks C, Marchini J. Meta-analysis and imputation refines the association of 15q25 with smoking quantity. *Nat Genet*. 2010; 42(5):436–440. [PubMed: 20418889]
- Thorgerirsson TE, Geller F, Sulem P, Rafnar T, Wiste A, Magnusson KP, Manolescu A, Thorleifsson G, Stefansson H, Ingason A, Stacey SN, Bergthorsson JT, Thorlacius S, Gudmundsson J, Jonsson T, Jakobsdottir M, Saemundsdottir J, Olafsdottir O, Gudmundsson LJ, Bjornsdottir G, Kristjansson K, Skuladottir H, Isaksson HJ, Gudbjartsson T, Jones GT, Mueller T, Gottsater A, Flex A, Aben KK, de Vegt F, Mulders PF, Isla D, Vidal MJ, Asin L, Saez B, Murillo L, Blondal T, Kolbeinnsson H, Stefansson JG, Hansdottir I, Runarsdottir V, Pola R, Lindblad B, van Rij AM, Dieplinger B, Haltmayer M, Mayordomo JI, Kiemeny LA, Matthiasson SE, Oskarsson H, Tyrfinngsson T, Gudbjartsson DF, Gulcher JR, Jonsson S, Thorsteinsdottir U, Kong A, Stefansson K. A variant associated with nicotine dependence, lung cancer and peripheral arterial disease. *Nature*. 2008; 452(7187):638–642. [PubMed: 18385739]
- Thorgerirsson TE, Gudbjartsson DF, Surakka I, Vink JM, Amin N, Geller F, Sulem P, Rafnar T, Esko T, Walter S, Gieger C, Rawal R, Mangino M, Prokopenko I, Magi R, Keskitalo K, Gudjonsdottir IH, Gretarsdottir S, Stefansson H, Thompson JR, Aulchenko YS, Nelis M, Aben KK, den Heijer M, Dirksen A, Ashraf H, Soranzo N, Valdes AM, Steves C, Uitterlinden AG, Hofman A, Tonjes A, Kovacs P, Hottenga JJ, Willemsen G, Vogelzangs N, Doring A, Dahmen N, Nitz B, Pergadia ML, Saez B, De Diego V, Lezcano V, Garcia-Prats MD, Ripatti S, Perola M, Kettunen J, Hartikainen AL, Pouta A, Laitinen J, Isohanni M, Huei-Yi S, Allen M, Krestyaninova M, Hall AS, Jones GT, van Rij AM, Mueller T, Dieplinger B, Haltmayer M, Jonsson S, Matthiasson SE, Oskarsson H, Tyrfinngsson T, Kiemeny LA, Mayordomo JI, Lindholt JS, Pedersen JH, Franklin WA, Wolf H, Montgomery GW, Heath AC, Martin NG, Madden PA, Giegling I, Rujescu D, Jarvelin MR, Salomaa V, Stumvoll M, Spector TD, Wichmann HE, Metspalu A, Samani NJ,

- Penninx BW, Oostra BA, Boomsma DI, Tiemeier H, van Duijn CM, Kaprio J, Gulcher JR, McCarthy MI, Peltonen L, Thorsteinsdottir U, Stefansson K. Sequence variants at CHRNA3-CHRNA6 and CYP2A6 affect smoking behavior. *Nat Genet.* 2010; 42(5):448–453. [PubMed: 20418888]
- Li MD, Xu Q, Lou XY, Payne TJ, Niu T, Ma JZ. Association and interaction analysis of variants in CHRNA5/CHRNA3/CHRNA4 gene cluster with nicotine dependence in African and European Americans. *Am J Med Genet B Neuropsychiatr Genet.* 2009
- Saccone NL, Wang JC, Breslau N, Johnson EO, Hatsukami D, Saccone SF, Gruzza RA, Sun L, Duan W, Budde J, Culverhouse RC, Fox L, Hinrichs AL, Steinbach JH, Wu M, Rice JP, Goate AM, Bierut LJ. The CHRNA5-CHRNA3-CHRNA4 nicotinic receptor subunit gene cluster affects risk for nicotine dependence in African-Americans and in European-Americans. *Cancer Res.* 2009; 69(17):6848–6856. [PubMed: 19706762]
- Baker TB, Weiss RB, Bolt D, von Niederhausern A, Fiore MC, Dunn DM, Piper ME, Matsunami N, Smith SS, Coon H, McMahon WM, Scholand MB, Singh N, Hoidal JR, Kim SY, Leppert MF, Cannon DS. Human neuronal acetylcholine receptor A5-A3-B4 haplotypes are associated with multiple nicotine dependence phenotypes. *Nicotine Tob Res.* 2009; 11(7):785–796. [PubMed: 19436041]
- Weiss RB, Baker TB, Cannon DS, von Niederhausern A, Dunn DM, Matsunami N, Singh NA, Baird L, Coon H, McMahon WM, Piper ME, Fiore MC, Scholand MB, Connett JE, Kanner RE, Gahring LC, Rogers SW, Hoidal JR, Leppert MF. A candidate gene approach identifies the CHRNA5-A3-B4 region as a risk factor for age-dependent nicotine addiction. *PLoS Genet.* 2008; 4(7):e1000125. [PubMed: 18618000]
- Saccone NL, Saccone SF, Hinrichs AL, Stitzel JA, Duan W, Pergadia ML, Agrawal A, Breslau N, Gruzza RA, Hatsukami D, Johnson EO, Madden PA, Swan GE, Wang JC, Goate AM, Rice JP, Bierut LJ. Multiple distinct risk loci for nicotine dependence identified by dense coverage of the complete family of nicotinic receptor subunit (CHRN) genes. *Am J Med Genet B Neuropsychiatr Genet.* 2009; 150B(4):453–466. [PubMed: 19259974]
- Chen X, Chen J, Williamson VS, An SS, Hettema JM, Aggen SH, Neale MC, Kendler KS. Variants in nicotinic acetylcholine receptors alpha5 and alpha3 increase risks to nicotine dependence. *Am J Med Genet B Neuropsychiatr Genet.* 2009; 150B(7):926–933. [PubMed: 19132693]
- Spitz MR, Amos CI, Dong Q, Lin J, Wu X. The CHRNA5-A3 region on chromosome 15q24-25.1 is a risk factor both for nicotine dependence and for lung cancer. *Journal of the National Cancer Institute.* 2008; 100(21):1552–1556. [PubMed: 18957677]
- Bierut LJ, Stitzel JA, Wang JC, Hinrichs AL, Gruzza RA, Xuei X, Saccone NL, Saccone SF, Bertelsen S, Fox L, Horton WJ, Breslau N, Budde J, Cloninger CR, Dick DM, Foroud T, Hatsukami D, Hesselbrock V, Johnson EO, Kramer J, Kuperman S, Madden PA, Mayo K, Nurnberger J Jr, Pomerleau O, Porjesz B, Reyes O, Schuckit M, Swan G, Tischfield JA, Edenberg HJ, Rice JP, Goate AM. Variants in Nicotinic Receptors and Risk for Nicotine Dependence. *Am J Psychiatry.* 2008; 165(9):1163–1171. [PubMed: 18519524]
- Sherva R, Kranzler HR, Yu Y, Logue MW, Poling J, Arias AJ, Anton RF, Oslin D, Farrer LA, Gelernter J. Variation in Nicotinic Acetylcholine Receptor Genes is Associated with Multiple Substance Dependence Phenotypes. *Neuropsychopharmacology.* 2010
- Greenbaum L, Kanyas K, Karni O, Merbl Y, Olender T, Horowitz A, Yakir A, Lancet D, Ben-Asher E, Lerer B. Why do young women smoke? I. Direct and interactive effects of environment, psychological characteristics and nicotinic cholinergic receptor genes. *Mol Psychiatry.* 2006; 11(3):312–322. 223. [PubMed: 16314871]
- Stevens VL, Bierut LJ, Talbot JT, Wang JC, Sun J, Hinrichs AL, Thun MJ, Goate A, Calle EE. Nicotinic receptor gene variants influence susceptibility to heavy smoking. *Cancer Epidemiol Biomarkers Prev.* 2008; 17(12):3517–3525. [PubMed: 19029397]
- Keskitalo K, Broms U, Heliovaara M, Ripatti S, Surakka I, Perola M, Pitkaniemi J, Peltonen L, Aromaa A, Kaprio J. Association of serum cotinine level with a cluster of three nicotinic acetylcholine receptor genes (CHRNA3/CHRNA5/CHRNA4) on chromosome 15. *Human molecular genetics.* 2009; 18(20):4007–4012. [PubMed: 19628476]
- Sherva R, Wilhelmsen K, Pomerleau CS, Chasse SA, Rice JP, Snedecor SM, Bierut LJ, Neuman RJ, Pomerleau OF. Association of a single nucleotide polymorphism in neuronal acetylcholine

- receptor subunit alpha 5 (CHRNA5) with smoking status and with 'pleasurable buzz' during early experimentation with smoking. *Addiction* (Abingdon, England). 2008; 103(9):1544–1552.
- Schlaepfer IR, Hoft NR, Collins AC, Corley RP, Hewitt JK, Hopfer CJ, Lessem JM, McQueen MB, Rhee SH, Ehringer MA. The CHRNA5/A3/B4 Gene Cluster Variability as an Important Determinant of Early Alcohol and Tobacco Initiation in Young Adults. *Biol Psychiatry*. 2008; 63(11):1039–1046. [PubMed: 18163978]
- Sasaki H, Hikosaka Y, Okuda K, Kawano O, Yukiue H, Yano M, Fujii Y. CHRNA5 Gene D398N Polymorphism in Japanese Lung Adenocarcinoma. *The Journal of surgical research*. 2009
- Le Marchand L, Derby KS, Murphy SE, Hecht SS, Hatsukami D, Carmella SG, Tiirikainen M, Wang H. Smokers with the CHRNA lung cancer-associated variants are exposed to higher levels of nicotine equivalents and a carcinogenic tobacco-specific nitrosamine. *Cancer research*. 2008; 68(22):9137–9140. [PubMed: 19010884]
- Amos CI, Wu X, Broderick P, Gorlov IP, Gu J, Eisen T, Dong Q, Zhang Q, Gu X, Vijayakrishnan J, Sullivan K, Matakidou A, Wang Y, Mills G, Doheny K, Tsai YY, Chen WV, Shete S, Spitz MR, Houlston RS. Genome-wide association scan of tag SNPs identifies a susceptibility locus for lung cancer at 15q25.1. *Nat Genet*. 2008; 40(5):616–622. [PubMed: 18385676]
- Hung RJ, McKay JD, Gaborieau V, Boffetta P, Hashibe M, Zaridze D, Mukeria A, Szeszenia-Dabrowska N, Lissowska J, Rudnai P, Fabianova E, Mates D, Bencko V, Foretova L, Janout V, Chen C, Goodman G, Field JK, Liloglou T, Xinarianos G, Cassidy A, McLaughlin J, Liu G, Narod S, Krokan HE, Skorpen F, Elvestad MB, Hveem K, Vatten L, Linseisen J, Clavel-Chapelon F, Vineis P, Bueno-de-Mesquita HB, Lund E, Martinez C, Bingham S, Rasmuson T, Hainaut P, Riboli E, Ahrens W, Benhamou S, Lagiou P, Trichopoulos D, Holcatova I, Merletti F, Kjaerheim K, Agudo A, Macfarlane G, Talamini R, Simonato L, Lowry R, Conway DI, Znaor A, Healy C, Zelenika D, Boland A, Delepine M, Foglio M, Lechner D, Matsuda F, Blanche H, Gut I, Heath S, Lathrop M, Brennan P. A susceptibility locus for lung cancer maps to nicotinic acetylcholine receptor subunit genes on 15q25. *Nature*. 2008; 452(7187):633–637. [PubMed: 18385738]
- Schwartz AG, Cote ML, Wenzlaff AS, Land S, Amos CI. Racial Differences in the Association Between SNPs on 15q25.1, Smoking Behavior, and Risk of Non-small Cell Lung Cancer. *J Thorac Oncol*. 2009
- Sherva R, Kranzler HR, Yu Y, Logue MW, Poling J, Arias AJ, Anton RF, Oslin D, Farrer LA, Gelernter J. Variation in nicotinic acetylcholine receptor genes is associated with multiple substance dependence phenotypes. *Neuropsychopharmacology*. 2010; 35(9):1921–1931. [PubMed: 20485328]
- Joslyn G, Brush G, Robertson M, Smith TL, Kalmijn J, Schuckit M, White RL. Chromosome 15q25.1 genetic markers associated with level of response to alcohol in humans. *Proc Natl Acad Sci U S A*. 2008; 105(51):20368–20373. [PubMed: 19064933]
- Gruzca RA, Wang JC, Stitzel JA, Hinrichs AL, Saccone SF, Saccone NL, Bucholz KK, Cloninger CR, Neuman RJ, Budde JP, Fox L, Bertelsen S, Kramer J, Hesselbrock V, Tischfield J, Nurnberger II Jr, Almasy L, Porjesz B, Kuperman S, Schuckit MA, Edenberg HJ, Rice JP, Goate AM, Bierut LJ. A risk allele for nicotine dependence in CHRNA5 is a protective allele for cocaine dependence. *Biological psychiatry*. 2008; 64(11):922–929. [PubMed: 18519132]
- Saccone SF, Hinrichs AL, Saccone NL, Chase GA, Konvicka K, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau O, Swan GE, Goate AM, Rutter J, Bertelsen S, Fox L, Fugman D, Martin NG, Montgomery GW, Wang JC, Ballinger DG, Rice JP, Bierut LJ. Cholinergic nicotinic receptor genes implicated in a nicotine dependence association study targeting 348 candidate genes with 3713 SNPs. *Hum Mol Genet*. 2007; 16(1):36–49. [PubMed: 17135278]
- Hoft NR, Corley RP, McQueen MB, Schlaepfer IR, Huizinga D, Ehringer MA. Genetic Association of the CHRNA6 and CHRNA3 Genes with Tobacco Dependence in a Nationally Representative Sample. *Neuropsychopharmacology*. 2009; 34(3):698–706. [PubMed: 18704094]
- Saccone NL, Culverhouse RC, Schwantes-An TH, Cannon DS, Chen X, Cichon S, Giegling I, Han S, Han Y, Keskitalo-Vuokko K, Kong X, Landi MT, Ma JZ, Short SE, Stephens SH, Stevens VL, Sun L, Wang Y, Wenzlaff AS, Aggen SH, Breslau N, Broderick P, Chatterjee N, Chen J, Heath AC, Heliovaara M, Hoft NR, Hunter DJ, Jensen MK, Martin NG, Montgomery GW, Niu T, Payne TJ, Peltonen L, Pergadia ML, Rice JP, Sherva R, Spitz MR, Sun J, Wang JC, Weiss RB, Wheeler W, Witt SH, Yang BZ, Caporaso NE, Ehringer MA, Eisen T, Gapstur SM, Gelernter J, Houlston

- R, Kaprio J, Kendler KS, Kraft P, Leppert MF, Li MD, Madden PA, Nothen MM, Pillai S, Rietschel M, Rujescu D, Schwartz A, Amos CI, Bierut LJ. Multiple independent loci at chromosome 15q25.1 affect smoking quantity: a meta-analysis and comparison with lung cancer and COPD. *PLoS Genet.* 2010; 6(8)
- Harris KM, Halpern CT, Smolen A, Haberstick BC. The National Longitudinal Study of Adolescent Health (Add Health) twin data. *Twin Res Hum Genet.* 2006; 9(6):988–997. [PubMed: 17254442]
- DeFries, JC.; Plomin, R.; Fulker, DW. Nature and nurture during middle childhood. Blackwell, Oxford, UK: Cambridge, Mass; 1994.
- Stallings MC, Corley RP, Dennehey B, Hewitt JK, Krauter KS, Lessem JM, Mikulich-Gilbertson SK, Rhee SH, Smolen A, Young SE, Crowley TJ. A genome-wide search for quantitative trait Loci that influence antisocial drug dependence in adolescence. *Arch Gen Psychiatry.* 2005; 62(9):1042–1051. [PubMed: 16143736]
- Elliott, DS.; Huizinga, D.; Menard, SW. Multiple problem youth : delinquency, substance use mental health problems. New York: Springer-Verlag; 1989.
- Sakai JT, Boardman JD, Gelhorn HL, Smolen A, Corley RP, Huizinga D, Menard S, Hewitt JK, Stallings MC. Using trajectory analyses to refine phenotype for genetic association: conduct problems and the serotonin transporter (5HTTLPR). *Psychiatric genetics.* 2010
- Anchordoquy HC, McGeary C, Liu L, Krauter KS, Smolen A. Genotyping of three candidate genes after whole-genome preamplification of DNA collected from buccal cells. *Behav Genet.* 2003; 33(1):73–78. [PubMed: 12645824]
- Zheng S, Ma X, Buffler PA, Smith MT, Wiencke JK. Whole genome amplification increases the efficiency and validity of buccal cell genotyping in pediatric populations. *Cancer Epidemiol Biomarkers Prev.* 2001; 10(6):697–700. [PubMed: 11401921]
- Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005; 21(2):263–265. [PubMed: 15297300]
- Laird NM, Lange C. Family-based designs in the age of large-scale gene-association studies. *Nat Rev Genet.* 2006; 7(5):385–394. [PubMed: 16619052]
- Lange C, DeMeo D, Silverman EK, Weiss ST, Laird NM. PBAT: tools for family-based association studies. *Am J Hum Genet.* 2004; 74(2):367–369. [PubMed: 14740322]
- Lange C, Silverman EK, Xu X, Weiss ST, Laird NM. A multivariate family-based association test using generalized estimating equations: FBAT-GEE. *Biostat %R* 101093/biostatistics/42195. 2003; 4(2):195–206.
- Laird NM, Horvath S, Xu X. Implementing a unified approach to family-based tests of association. *Genet Epidemiol.* 2000; 19(Suppl 1):S36–S42. [PubMed: 11055368]
- Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet.* 2004; 74(4):765–769. [PubMed: 14997420]
- Purcell SM, Wray NR, Stone JL, Visscher PM, O'Donovan MC, Sullivan PF, Sklar P. Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature.* 2009; 460(7256):748–752. [PubMed: 19571811]
- Wang JC, Grucza R, Cruchaga C, Hinrichs AL, Bertelsen S, Budde JP, Fox L, Goldstein E, Reyes O, Saccone N, Saccone S, Xuei X, Bucholz K, Kuperman S, Nurnberger J Jr, Rice JP, Schuckit M, Tischfield J, Hesselbrock V, Porjesz B, Edenberg HJ, Bierut LJ, Goate AM. Genetic variation in the CHRNA5 gene affects mRNA levels and is associated with risk for alcohol dependence. *Molecular psychiatry.* 2009; 14(5):501–510. [PubMed: 18414406]
- Wang JC, Cruchaga C, Saccone NL, Bertelsen S, Liu P, Budde JP, Duan W, Fox L, Grucza RA, Kern J, Mayo K, Reyes O, Rice J, Saccone SF, Spiegel N, Steinbach JH, Stitzel JA, Anderson MW, You M, Stevens VL, Bierut LJ, Goate AM. Risk for nicotine dependence and lung cancer is conferred by mRNA expression levels and amino acid change in CHRNA5. *Human molecular genetics.* 2009; 18(16):3125–3135. [PubMed: 19443489]

Fig. 1.

Mean symptom count versus genotype for SNP rs8040868 in the Add Health sample. C is the minor allele for rs8040868.

Fig. 2.
Mean symptom count versus genotype for SNP rs8040868 in the CADD sample. C is the minor allele for rs8040868.

Fig. 3.
Correlations between the composite of SNP rs8040868 and the individual item scores for all 18 items in the Add Health sample.

Table 1

Haploview results for SNPs utilized for this study.

SNP	Location	Gene	Alleles Major/Minor	Minor Allele Frequency			Hardy-Weinberg P-value	
				Add Health (call rate %)	CADD (call rate %)	NYS-FS (call rate %)	Addhealth	CADD
rs684513	intron 1	CHRNA5	C/G	0.22(91)	0.23(96)	0.21(84)	0.002	0.761
rs680244	intron 1	CHRNA5	C/T	0.41(86)	0.43(97)	0.46(81)	0.639	0.219
rs16969968	exon 5	CHRNA5	G/A	0.31(97)	0.32(81)	0.32(82)	0.309	0.966
rs514743	intron 5	CHRNA5	A/T	0.37(94)	0.36(98)	0.39(82)	0.105	0.279
rs11637630	intron 3	CHRNA3	A/G	0.26(94)	0.24(97)	0.24(82)	0.0001	0.266
rs8040868	exon 2	CHRNA3	T/C	0.37(92)	0.39(97)	0.39(79)	0.047	0.803
rs8023462	intergenic	CHRNA3/B4	T/C	0.36(86)	0.36(97)	0.38(81)	0.007	0.739
rs1948	UTR-3'	CHRNA4	G/A	0.34(93)	0.35(98)	0.36(84)	0.473	0.963
rs1316971	intron 1	CHRNA4	G/A	0.24(84)	0.21(93)	0.21(83)	0.0003	0.282
rs11634351	upstream	CHRNA4	G/A	0.38(88)	0.4(97)	0.42(82)	0.351	0.329

Table II

Results of association studies with selected SNPs in the *CHRNA5/CHRNA3/CHRNA4* gene cluster and externalizing behaviors. Family-based tests of association in the Add Health and NYS-FS samples, were carried out with FBAT-PC approach (Lasky-Su et al. 2008).

SNP	Gene	Add Health N=1476	CADD N=730	NYS-FS N=865	Saccone et al. (2010) Independent Locus
rs684513	<i>CHRNA5</i>	0.06	0.596	0.205	N/A
rs680244	<i>CHRNA5</i>	0.673	0.212	0.049	Locus 3
rs16969968	<i>CHRNA5</i>	0.119	0.238	0.05	Locus 1
rs514743	<i>CHRNA5</i>	0.245	0.514	0.63	N/A
rs11637630	<i>CHRNA3</i>	–	0.831	0.586	Locus 2
rs8040868	<i>CHRNA3</i>	0.04	0.007*	–	N/A
rs8023462	<i>CHRNA3/CHRNA4</i>	0.671	0.018	–	N/A
rs1948	<i>CHRNA4</i>	0.633	0.033	0.565	N/A
rs1316971	<i>CHRNA4</i>	–	0.726	0.746	N/A
rs11634351	<i>CHRNA4</i>	0.054	0.161	0.18	N/A

Family-based tests of association in the Add Health and NYS-FS samples, were carried out with FBAT-PC approach (Lasky-Su et al. 2008). For family-based analyses, cigarettes per day (CPD), sex, age, and age² were utilized as covariates. In the CADD sample linear regression was employed to analyze CD lifetime symptom count after transformation described in the text.

* P-value meets threshold for significance estimated by the SNPSpD program (Nyholt, 2004). Dashes indicate the SNP was excluded from the analyses because it did not conform to Hardy-Weinberg proportions in this sample with a threshold HWE p-value ≤ 0.001 . Column six shows results of the current study mapped onto the distinct nicotine dependence association signals in the *CHRNA5/CHRNA3/CHRNA4* gene cluster as described by Saccone et al. 2009 and Saccone et al. 2010.