ORIGINAL INVESTIGATION

Effect of Neuronal Nicotinic Acetylcholine Receptor Genes (*CHRN*) on Longitudinal Cigarettes per Day in Adolescents and Young Adults

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ABSTRACT

Introduction: Few studies have sought to identify specific genetic markers associated with cigarettes per day (CPD) during adolescence and young adulthood, the period of greatest vulnerability for the development of nicotine dependence.

Methods: We used a longitudinal design to investigate the effect of neuronal nicotinic acetylcholine receptor (*CHRN*) subunit genes on CPD from 15 to 21 years of age in young smokers of European descent ($N = 439$, 59% female). The number of CPD typically smoked during the previous 30 days was self-reported. Single nucleotide polymorphisms (SNPs) from *CHRN* genes were genotyped using DNA extracted from saliva samples collected at the 5-year assessment. Mixed-model analyses of SNP effects were computed across age at the time of assessment using log-transformed CPD as the phenotype. Data from the 1000 Genomes Project were used to clarify the architecture of *CHRN* genes to inform SNP selection and interpretation of results.

Results: CPD was associated with a *CHRNB3A6* region tagged by rs2304297, with *CHRNA5A3B4* haplotype C (tagged by rs569207), and with the *CHRNA2* SNP rs2271920, $ps < .004$. The reliability of single-SNP associations was supported by the correspondence between a more extensive set of SNP signals and the underlying genetic architecture. The 3 signals identified in this study appear to make independent contributions to CPD, and their combined effect accounts for 5.5% of the variance in log-transformed CPD.

Conclusions: Level of CPD during adolescence and young adulthood is associated with *CHRNB3A6, CHRNA5A3B4*, and *CHRNA2*.

INTRODUCTION

Adolescence through young adulthood is the critical age for cigarette smoking initiation and the development of nicotine dependence (ND) (Eaton et al., 2012; U.S. Department of Health and Human Services, 2012). Significant advances have been made in our understanding of nongenetic factors associated with smoking during this developmental period (Audrain-McGovern et al., 2012; Chassin, Presson, Rose, & Sherman, 1996; Mermelstein, Hedeker, & Weinstein, 2009; Selya et al., 2013; Zhan, Dierker, Rose, Selya, & Mermelstein, 2012), but few studies have sought to uncover specific genetic risk factors for postinitiation smoking by youth. This knowledge gap contrasts with advances in the identification of specific genetic markers for ND in adults (Benowitz, 2009) and the progress made with smoking initiation phenotypes (Ehringer et al., 2007, 2010; TAG Consortium, 2010; Thorgeirsson et al., 2010; Zeiger et al., 2008). A better understanding of the genetic variables that affect youth smoking may lead to more effective prevention and smoking cessation interventions for them.

Neuronal nicotinic acetylcholine receptor (*CHRN*) subunit genes, which encode nicotinic acetylcholine receptors (nAChRs) (Mineur & Picciotto, 2008), constitute a promising family of genes to query regarding smoking progression because most have been implicated in ND severity in cross-sectional studies of adult smokers (Greenbaum & Lerer, 2009; Saccone et al., 2009). The strongest evidence for *CHRN* gene association with adult ND has been reported for the *CHRNA5A3B4* and the *CHRNB3A6* clusters (TAG Consortium, 2010; Thorgeirsson et al., 2010). These clusters include *CHRNA5* and *CHRNA6*, which encode the α5 and α6 nAChR subunits that are differentially associated with

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nicotine's aversive and appetitive effects, respectively, in animal studies (Brunzell, 2012; Brunzell, Boschen, Hendrick, Beardsley, & McIntosh, 2010; Exley, Clements, Hartung, McIntosh, & Cragg, 2008; Fowler & Kenny, 2012; Fowler, Lu, Johnson, Marks, & Kenny, 2011). Specifically, α6β2* receptors are essential for nicotine appetitive reinforcement (Brunzell, 2012; Brunzell et al., 2010; Exley et al., 2008). In contrast, α5* receptors have no effect on appetitive nicotine reinforcement at low doses but inhibit administration of high doses (Fowler & Kenny, 2012; Fowler et al., 2011).

Resequencing of the *CHRNA5A3B4* region in adults identified a linkage disequilibrium (LD) block with four common haplotypes in a cohort of European descent (Weiss et al., 2008). This LD block includes *CHRNA5* and *CHRNA3* exonic regions, with haplotype A (HA, most frequent) tagged by the minor allele at rs16969968 or rs1051730, HB by rs680244 or rs514743, HC by rs569207 or rs578776, and HD (least frequent) by rs11633585. In adults, HA is associated with greater ND severity than is HB, and HB is associated with greater ND severity than is HC (Baker et al., 2009; Berrettini et al., 2008; Liu et al., 2010; Saccone et al., 2010; TAG Consortium, 2010; Weiss et al., 2008). The low frequency of HD (≈0.05 in European populations) has impeded characterization of its phenotypic effects. Association of single nucleotide polymorphisms (SNPs) in the *CHRNB3A6* cluster with adult smoking phenotypes is consistent with a strong LD block centered on the *CHRNB3* promoter region (Saccone et al., 2007; Thorgeirsson et al., 2010) and with weaker LD extending SNP association into the *CHRNA6* region.

In contrast to the evidence of robust effects of *CHRN* genes on ND severity, *CHRN* genes were not associated with ever having smoked (TAG Consortium, 2010; Thorgeirsson et al., 2010) and the evidence with respect to age of first cigarette is mixed (Caporaso et al., 2009; Schlaepfer et al., 2008). *CHRNA5A3B4* haplotypes were not associated with age of daily smoking, but HA was more strongly associated with ND in adults who began daily smoking by age 16 years (Baker et al., 2009; Hartz et al., 2012; Weiss et al., 2008).

Two longitudinal studies report *CHRNA5A3B4* HA tag SNP associations with smoker category (current or heavy/regular smoker vs. past or never smoker) at early adolescence (ages 13–15) and later at either age 18 (Rodriguez et al., 2011) or age 31 (Ducci et al., 2011). Ducci et al. (2011) observed a moderate association of both HA and HB tag SNPs in high-quantity smokers compared to low-quantity/nonsmokers at age 14, and the association of HA tag SNPs strengthened while the HB tag SNPs weakened at age 31, consistent with previous findings in adult smokers (Saccone et al., 2010). Rodriguez et al. (2011) also observed an HA tag SNP association at age 13–15 and at age 18. No association was reported between smoking behavior and the HC tag SNP rs578776 (Rodriguez et al., 2011) or the HC tag SNP rs6495309 (Ducci et al., 2011).

Here, we report the effects of *CHRN* genes on cigarettes per day (CPD) assessed longitudinally in young non-Hispanic Whites who smoked at least one puff. Thus, this is a study of genetic markers for early postinitiation smoking. Our cohort comprised primarily light, infrequent smokers, a very different phenotype than that typical of adult ND studies. This study also extends prior longitudinal studies of *CHRNA5A3B4* effects on smoking in young people (Ducci et al., 2011; Rodriguez et al., 2011) by surveying other neuronal *CHRN* genes. The *CHRNB3A6* and *CHRNA5A3B4* gene clusters were of particular a priori interest due to their robust effects in both preclinical and adult association studies, so these gene clusters were more densely surveyed.

METHODS

Participants

The sample was drawn from the Social and Emotional Contexts of Adolescent Smoking Patterns (SECASP) Study (Dierker & Mermelstein, 2010; Selya et al., 2013). The SECASP cohort (N = 1,263) was recruited from 12,970 9th and 10th graders in the Chicago area who were screened regarding tobacco use. The SECASP cohort was representative of the Chicago metropolitan area in race and ethnicity (56% non-Hispanic Whites, 17% non-Hispanic African Americans, 13% Hispanic Whites, and 14% all other categories). Because the overarching aim of the SECASP project is to study smoking progression rather than either smoking initiation or ND, students who had ever smoked but were not yet regular smokers were oversampled (see Supplementary Material for details regarding participant accession criteria).

SECASP participants were followed longitudinally into young adulthood, with assessments at baseline, 6, 15, 24, 33 months, and 4 and 5 years. Participants contacted during Year 5 (N = 1,027 [81% of total cohort]) were asked to participate in the genetic arm of the project and provide a saliva sample for DNA extraction; 953 (93%) of those asked agreed. To reduce population stratification effects (Pritchard & Rosenberg, 1999), we limited the current analyses to non-Hispanic Whites. To focus on smoking progression rather than initiation, we included only those who had smoked at least one cigarette puff between baseline and Year 5. Of the 439 participants in the final analytic sample, 59% were female. At baseline, their mean age was 15.7 years (SD = 0.60, range = 13.9–17.0). At Year 5, their mean age was 21.4 years (SD = 0.79, range = 19.2–23.3).

CPD Phenotype

At each assessment, participants were asked how many CPD they typically smoked on days smoked during the previous 30 days and how many of the previous 30 days they had smoked. To permit analysis of age effects, these measures were binned on the basis of age in years at the time of assessment: 15 or less, 16, 17, 18–19, and 20 or more. Occasionally, a participant had more than one assessment within an age bin (e.g., both the baseline and 6-month observations may have occurred within age 16): these observations were averaged within that bin. A total of 1,987 observations were available across age bins (number of observations by age bin: 15 or less, 282; 16, 427; 17, 424; 18–19, 431; and 20+, 423).

nAChR Marker Selection

We chose tag SNP markers (cf. Supplementary Table S1 for a complete list) from the *CHRNA5A3B4* and *CHRNB3A6* gene clusters and the *CHRNA4*, *CHRNB2*, and *CHRNA2* genes; *CHRNA7* was not surveyed due to complications the *CHRFAM7A* chimera causes in interpreting biallelic genotypes, and *CHRNA9* and *10* were not surveyed due to their limited central nervous system expression and lack of association with adult ND phenotypes. A SNP pairwise correlation

squared (r^2) threshold of >0.64 and a minor allele frequency of >0.05 were used to group SNPs into LD bins (Carlson et al., 2004) using 261 of the 371 European 1000 Genomes Phase 1 unrelated individuals. Tag SNP selection was further refined by including potentially functional variants such as the *CHRNA5* nonsynonymous SNP rs16969968 and the *CHRNB2* 3′ untranslated region SNP rs2072661 (Conti et al., 2008). Additional markers were added to capture in finer detail the LD structures of the *CHRNA5A3B4* and *CHRNB3A6* gene clusters. DNA collection, genotyping methods, and methods to impute genotypes and infer haplotypes from the directly genotyped tag SNPs are described in the Supplementary Material.

Data Analyses

Because CPD was not normally distributed, we employed a $\log_{10}(\text{CPD} + 1)$ transformation of CPD, designated LOG(CPD). Additive SNP coding was used. A linear mixed model for longitudinal data (Hedeker & Gibbons, 2006), with random subject intercepts and trends across age, was used to test the following model:

$$\text{LOG(CPD)} = \text{intercept} + \text{age} + \text{sex} + \text{SNP}$$

This model tested age and SNP effects across time. Sex was a covariate because males increased CPD at a steeper rate and reached a higher level of CPD than did females (cf. Results). Analyses of SNP interactions with age and sex were not significant, so only SNP main effects across age and sex are reported. We calculated effect sizes (ESs) for SNP main effects based on the mixed-model estimates. These ESs represent mean differences in LOG(CPD) per minor allele, divided by the *SD* of LOG(CPD). Because this *SD* varies across ages in the mixed model with random intercepts and age effects, we estimated the ES at each age and present the averaged ES across ages. A Bonferroni *p*-value adjustment was computed separately for each gene or gene cluster, in which the number of tests is reduced based on the LD of SNPs within a gene or gene cluster (Galwey, 2009) (see Supplementary Material). Supplementary Table S1 contains results of tests of SNP associations with log-transformed days smoked, which were comparable to those for LOG(CPD).

RESULTS

CPD by Age and Sex

LOG(CPD) increased with age across sexes, $p < .0001$, and males smoked more across ages, $p < .009$. The sex difference became greater with age, $p < .02$. For females, CPD increased from 1.0 (*SD* = 1.5) at age 15 to 4.1 (*SD* = 5.1) at age 20; for males, CPD increased from 1.4 (*SD* = 2.1) to 6.0 (*SD* = 6.2) at those two age bins.

CHRNB3A6 Markers

Multiple *CHRNB3A6* SNPs were associated with LOG(CPD), *p*s < .009 (Table 1). The relations between ESs and LD with rs2304297 and rs4950 suggest a single genetic signal in the rs2304397-tagged LD bin. The correlation between LD with rs2304297 and absolute ES values for LOG(CPD) (Table 1) was 0.83, $p < .0008$. By contrast, ES correlation with rs4950 LD was only 0.36, $p = .26$. The strong relation between rs2304297 LD and ES argues against significant *CHRNB3A6*-phenotype associations due to Type I errors for single SNPs. The minor allele of rs2304297 was associated with lower CPD (Supplementary Figure S1). The association of imputed SNPs and the haplotype structures spanning the region (Supplementary Figure S2) suggest that this multi-SNP signal derives from a haplotype that differs subtly between the *CHRNB3* and *CHRNA6* end of the cluster, with the association signal strengthened by the pattern of minor alleles at *CHRNA6*.

Table 1. *CHRNB3A6* SNP Associations With LOG(CPD)

		LOG(CPD)		LD (r^2)	
LD bin	SNP	*p* values	ES	rs2304297	rs4950
BIN02	rs2304297	**.003**	−0.19	1.00	0.48
BIN04	rs7017612	.01	−0.18	0.57	0.54
BIN02	rs6982753	.01	−0.17	0.80	0.43
BIN02	rs892413	.01	−0.17	0.82	0.37
BIN01	rs6474412	**.008**	−0.17	0.46	0.98
BIN01	rs4950	.02	−0.17	0.48	1.00
BIN03	rs11986893	**.009**	−0.17	0.78	0.31
BIN02	rs10107450	**.009**	−0.16	0.94	0.45
BIN03	rs4737071	.01	−0.16	0.77	0.31
BIN08	rs28611189	.13	−0.11	0.27	0.56
BIN01	rs1530847	.12	−0.10	0.34	0.59
BIN09	rs17621710	.36	−0.08	0.04	0.00

Note. CPD = cigarettes per day; ES = effect size; LD = linkage disequilibrium; SNP = single nucleotide polymorphism. Both *p* values and ESs are shown; *p* values < 0.01 are shown in bold. SNPs are sorted in descending order by ES for LOG(CPD). The last two columns are the LD (r^2) in this sample of each SNP with rs2304297 and rs4950, respectively. LD bins are based on an r^2 criterion of 0.64.

CHRNA5A3B4 Markers

Two HC tag SNPs (rs569207 and rs12443170) were significant (*p*s < .004), but no tag SNPs for HA, HB, or HD were significant (Table 2; see Supplementary Table S1 for results for other *CHRNA5A3B4* markers). The minor allele of both HC SNPs was associated with lower CPD (rs569207 shown in Supplementary Figure S3). Association with CPD of imputed SNPs and haplotype clustering (Supplementary Figure S4) increases confidence in the reliability of the observed HC effect. The absence of signals for HA and HB tag SNPs reflects the small ESs of HA and HB SNPs relative to those of HC SNPs (Table 2). The absence of an association between rs16969968 and CPD is illustrated in Supplementary Figure S5.

To parse the effects of *CHRNA5A3B4* variants controlling for homozygous and heterozygous alleles, a mixed model was tested in which the diplotype (haplotype pairs on each chromosome, akin to a multilocus genotype) effect was the genotypic variable. The low-frequency D* diplotypes were excluded, as were observations in which one of the haplotypes was either uncalled or a rare variant other than HA–HD (Supplementary Figure S6). Finding a significant diplotype main effect across age, $p = .004$, all possible pairwise contrasts were tested. These tests indicated that diplotypes AB, AC, and BB were associated

Table 2. *CHRNA5A3B4* Associations With LOG(CPD) for Haplotype Tag SNPs

		LOG(CPD)	
Haplotype	SNP	*p* values	ES
HA	rs1051730	.24	0.07
HA	rs16969968	.37	0.05
HB	rs514743	.24	0.07
HB + HD	rs680244	.07	0.10
HC	rs569207	**.001**	−0.20
HC (sub)	rs12443170	**.002**	−0.25
HC + HD	rs578776	.06	−0.11
HD	rs11633585	.11	0.19

Note. CPD = cigarettes per day; ES = effect size; SNP = single nucleotide polymorphism. Both *p* values and ESs are shown; *p* values < 0.01 are shown in bold. SNPs are sorted by haplotype. SNPs that the 1000 Genomes Project genetic architecture (see Figure 2) indicate tag more than one haplotype (rs680244 and rs578776) or a subset of a haplotype (rs12443170) are so indicated in the haplotype column.

Table 3. *p* Values for Pairwise Contrasts of *CHRNA5A3B4* Diplotypes

	AA	AB	AC	BB	BC
AB	.15				
AC	.43	.49			
BB	.23	.98	.59		
BC	.41	**.003**	**.04**	**.02**	
CC	.10	**.005**	**.02**	**.01**	.20

Note. The diplotype main effect was significant, $p = .004$. In post-hoc mixed-model analyses, all pairwise contrasts were tested. Nominally significant *p* values (<.05) are shown in bold font.

with higher LOG(CPD) than were either BC or CC (Figure 1 and Table 3).

CHRNA2, *CHRNA4*, and *CHRNB2* Markers

A significant association was obtained for one *CHRNA2* SNP, rs2271920, $p = .004$, ES = 0.16. The minor allele of rs2271920 was associated with higher CPD (Supplementary Figure S7). Imputed SNPs tagging the same haplotype cluster as rs2271920 showed similar *p* values (Supplementary Figure S8), supporting the reliability of the rs2271920 signal. No other *CHRNA2* SNP nor any *CHRNA4* and *CHRNB2* SNPs reached within-gene significance (Supplementary Table S1).

CHRNB3A6, *CHRNA5A3B4*, and *CHRNA2* Joint Effects

We next examined the joint effects of rs2304297, rs569207, and rs2271920. In a series of analyses in which all three-way and two-way SNP interactions were tested, no significant SNP × SNP interaction effects were obtained. In a final model containing all three SNPs but no interaction terms, all SNPs were significant, *p*s < .008. Thus, these three SNPs appear to make *independent* contributions to the prediction of LOG(CPD). Finally, to assess the *combined* effects of the three SNPs, the net number of protective alleles was computed as rs2304297 + rs569207 − rs2271920. The main effect of net protective alleles was highly significant, $p < .0001$, and an r^2 analysis indicated the net number of protective alleles accounted for 5.5% of LOG(CPD) variance. As shown in Supplementary Figure S9, there was an inverse ordinal relation between net protective alleles and LOG(CPD) across ages.

DISCUSSION

In young, light smokers, a survey of *CHRN* genes found associations between CPD and *CHRNA5A3B4, CHRNB3A6,* and *CHRNA2* but no associations with *CHRNA4* or *CHRNB2*. The *CHRNB3A6* signal was best tagged by rs2304297, the *CHRNA5A3B4* signal by HC (particularly rs569207), and the *CHRNA2* signal by rs2271920. The effects of rs2304297, rs569207, and rs2271920 appear to be independent, and their combined effect accounted for 5.5% of the LOG(CPD) variance. Although methodological and computational differences require caution in making a direct comparison with other studies, in analyses of two adult datasets rs16969968 accounted for 1.22% and 0.87% of the variance in Fagerström Test for Nicotine Dependence-defined case/control status (Culverhouse et al., 2011). The reliability of these 3 signals is supported by their relations with genetic architecture (LD, imputed SNPs, or haplotype). Previous studies of *CHRN* effects on smoking progression investigated only *CHRNA5A3B4* SNPs (Ducci et al., 2011; Rodriguez et al., 2011). To our knowledge, our report is the first to find smoking progression effects for *CHRNB3A6* and *CHRNA2*.

The strong signals from *CHRNA5A3B4* and *CHRNB3A6* are consistent with the evidence from animal studies of the importance of α5* and α6*nAChR receptors in nicotine's aversive and appetitive effects (Brunzell, 2012; Brunzell et al., 2010; Exley et al., 2008; Fowler & Kenny, 2012; Fowler et al., 2011) and with studies in adult smokers that indicate stronger effects for *CHRNA5A3B4* and *CHRNB3A6* than for other *CHRN* genes (TAG Consortium, 2010; Thorgeirsson et al., 2010). These findings extend the evidence for the prepotency of *CHRNA5A3B4* and *CHRNB3A6* to a population of smokers whose smoking heaviness is substantially less.

Previous studies (Ducci et al., 2011; Rodriguez et al., 2011) of the longitudinal effect of *CHRNA5A3B4* on smoking in youth found single-SNP associations with HA tag SNPs, but we did not. Given the exceptionally strong evidence that HA is associated with heavier smoking in adults (Bierut et al., 2008; Liu et al., 2010; Saccone et al., 2010; TAG Consortium, 2010; Thorgeirsson et al., 2010; Weiss et al., 2008), the lack of an association between single HA SNPs and smoking heaviness in our study was unexpected. However, the contrast between the small ESs of HA and HB SNPs and the larger ESs of HC SNPs (Table 2) does not support the conclusion that we missed a substantial 'true' signal due to insufficient power. It is possible that our null finding is attributable to low nicotine dose. Smoking heaviness was much lower in our cohort than is typical in adult studies that have reliably shown HA effects and was lower than in previous adolescent studies (Ducci et al., 2011; Rodriguez et al., 2011). The animal literature suggests α5* nAChR receptors do not block the appetitive effects of low nicotine doses but rather affect the response to aversive nicotine effects at higher doses (Fowler & Kenny, 2012; Fowler et al., 2011). That the human haplotype A may be particularly sensitive to

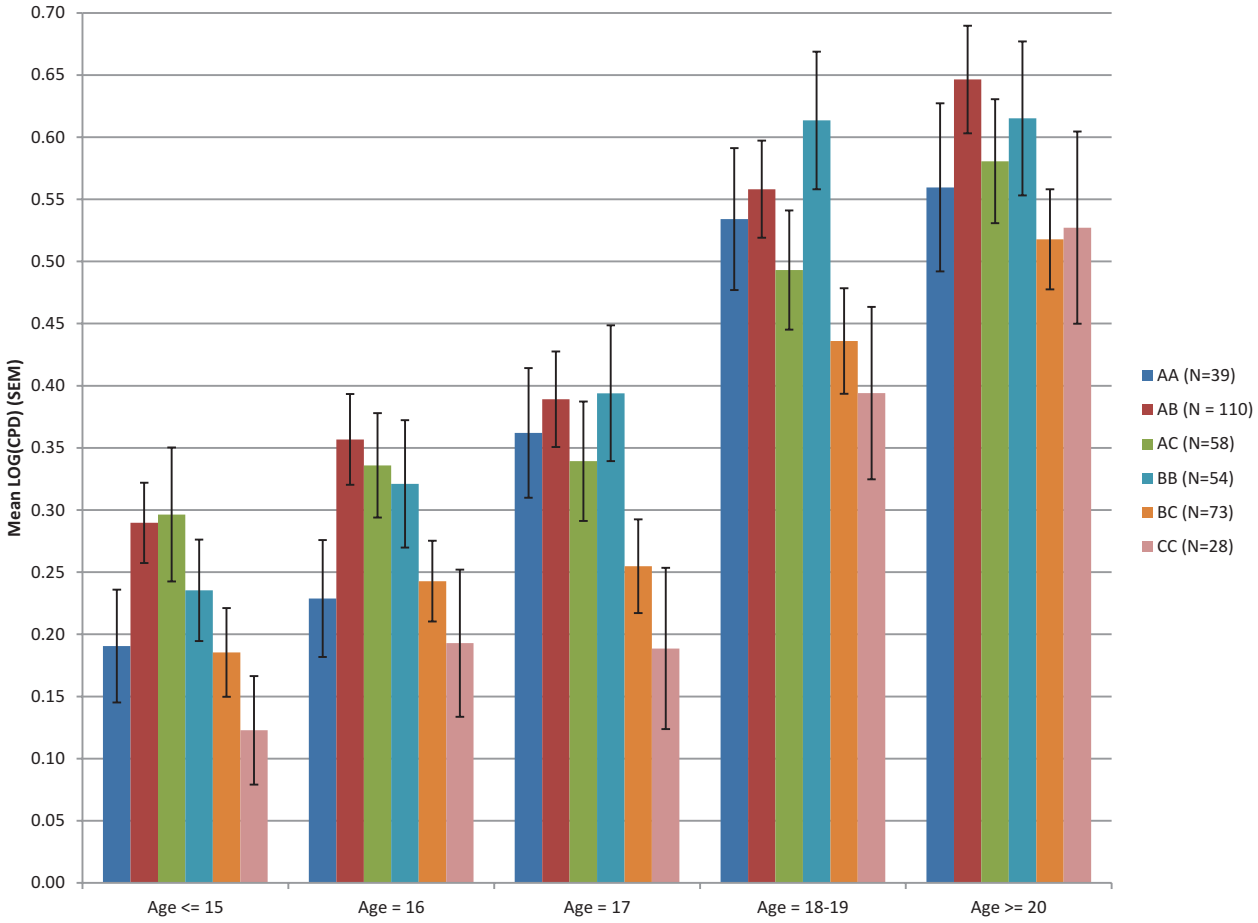

Figure 1. Mean LOG(CPD) by age and *CHRNA5A3B4* diplotype. The legend shows the mean number of observations per diplotype and age across age bins from 16 to 20+. Note that D* diplotypes were excluded due to their low frequency.

nicotine dose is suggested by the report that lentiviral-mediated medial habenula expression of the mouse homolog of the rs16969968 α5 D398N risk allele reduces aversive nicotine effects (Frahm et al., 2011). Other possible explanations of the null HA effect are offered in the Supplementary Material.

Neither our study nor that of Rodriguez et al. (2011) found a significant effect for the *CHRNA5A3B4* SNP rs578776, but we did find a significant effect for the HC tag SNP rs569207, a SNP not studied by Rodriguez et al. (2011). Because rs569207 more effectively parses HC and HD than does rs578776 (see Supplementary Figure S4), the stronger effect of rs569207 is consistent with functional variants residing within HC. The finding that rs12443170 retains a strong association signal while sub-dividing the HC haplotype requires further investigation. rs12443170 is within intron 4 of *CHRNA3* and is adjacent to rs6495308 (a full HC tag, 80 bp 5′), which was the second-most significant imputed *CHRNA5A3B4* SNP association for CPD in a study of adults (Liu et al., 2010) after controlling for HA association. Unlike rs569207, which is more frequent in populations of Asian and African ancestry, rs12443170 is more frequent in European populations, indicating that further investigation of sub-divisions of the HC haplotype is warranted.

Our single-SNP results for *CHRNA5A3B4* are clarified by diplotype contrasts, which suggest that both HA and HB are associated with higher CPD than is HC, with HA and HB not differing from each other. Assuming additive haplotype effects (Saccone et al., 2010; Weiss et al., 2008), the AB and BB contrasts with BC and CC clearly suggest that HB was associated with higher LOG(CPD) than was HC. The results for A* diplotypes are complicated by the absence of significant AA contrasts with other diplotypes, but those null findings could be due to the small number of observations for AA (n = 39; Figure 1). However, assuming additive haplotype effects, the significant AB versus BC and the AC versus CC contrasts support the conclusion that HA is associated with heavier smoking than is HC. Alternatively, rather than additive haplotype effects, it may be that there is a complex dominance model of haplotype effects. It is not yet possible to decide between these two inheritance models. The absence of an HA versus HB effect in our results is consistent with the equivalence at age 14 of HA and HB 'risk' effects reported by Ducci et al. (2011). We encourage further research on HA and HB effects at low nicotine doses, especially among young people in early stages of smoking.

In this study, the *CHRNB3A6* signal was better tagged by rs2304297 than by rs4950 or rs6474412. *CHRNB3* variants in LD with rs2304297 have reached genome-wide significance in multiple large association studies with adult CPD and Fagerström Test for Nicotine Dependence phenotypes (Rice et al., 2012; Thorgeirsson et al., 2010). The largest ESs in adult studies were seen with SNPs in a strong ($r^2 > 0.95$) European LD bin centered on the promoter region of *CHRNB3* that

includes rs4950, rs6474412, rs6474413, and rs1451240. In two studies (Saccone et al., 2007; Thorgeirsson et al., 2010), rs2304297 had lower ESs than the rs4950 LD bin. However, in one adult study using *DSM-IV* ND, rs2304297 had a higher ES than rs4950 (Hoft et al., 2009). Previous studies in adolescents have detected stronger associations of the rs4950 LD bin and weaker associations of the rs2304297 LD bin with subjective response to tobacco (e.g., buzz or dizziness), but neither LD bin was associated with measures of use (Zeiger et al., 2008). rs2304297 is located in the *CHRNA6* 3′ untranslated region 125 nucleotides downstream of the stop codon, and examination of the *CHRNB3A6* haplotypic structure (see Supplementary Figure S3) suggests that the rs2304397-tagged LD bin extends across the *CHRNA6* region, distinct from the rs4950-tagged LD bin across the *CHRNB3* region. The contrasting ESs of rs2304297 and rs4950 in our study versus adult cohorts suggest that multiple common functional variants may be segregating at the *CHRNB3A6* cluster.

The association of rs2271920 in the *CHRNA2* region is novel, but the signal is further supported by imputed SNPs that extend a haplotype cluster into the flanking gene, *PTK2B*.

Limitations

One limitation is a relatively small sample size ($N = 439$). To assess the likelihood that this study was underpowered, we calculated the post-hoc power (Hedeker, Gibbons, & Waternaux, 1999) of two of the statistically significant SNPs (rs2304297 and rs569207) using parameter estimates from mixed models in which SNPs were coded as dominant effects to simplify power estimation. Power equaled 0.73 and 0.67 for these two SNPs, respectively. Although not high, these represent reasonable levels of statistical power for genetic effects of the magnitude we did detect. However, post-hoc power calculations do not always provide accurate estimates, especially if true power is low (Yuan & Maxwell, 2005). Thus, for weaker genetic effects, power may have been <0.70. In addition, we attempted to control Type I errors by limiting our analyses to genes for which there is substantial a priori evidence of effects on other smoking phenotypes, correcting for multiple testing within genes, and assessing the relation among significant *p*-values within a gene and the gene's underlying architecture. Thus, we are confident that our positive findings are replicable, but we cannot rule out the possibility that we missed some true genetic effects.

Another limitation is that we included only non-Hispanic Whites in our analyses, so the results may not generalize to other race/ethnicity groups.

It is possible that the study accession criterion that excluded students who were regular smokers at baseline may have biased the cohort in a way that diminished *CHRNA5A3B4* HA effects that would have been seen in a population-based sample. The number of potential participants excluded by this criterion is so small (0.9%) that we believe such bias to be unlikely, but we discuss this possibility further in the Supplementary Material. It is also possible that the oversampling of ever-smokers may have altered *CHRN** allele frequencies relative to a population sample, but the lack of evidence that *CHRN** genes are associated with smoking initiation does not support this possibility (TAG Consortium, 2010; Thorgeirsson et al., 2010). Further, even if allele frequencies did differ from those of the population, such a shift would not be relevant to our purpose, which was to identify genetic associations with smoking progression in youth who had ever smoked rather than with smoking initiation.

Our primary outcome (CPD) is limited by self-report; but at low levels of smoking among adolescents, there are few valid alternatives (Mermelstein et al., 2002). Further, self-reported CPD was consistent with other measures of smoking level in our study (e.g., other survey questions asking about smoking quantity in different ways as well as in-person timeline follow-back interviews).

CONCLUSIONS

We report independent effects of SNPs from *CHRNB3A6, CHRNA5A3B4*, and *CHRNA2* on CPD in smokers 15–21 years of age, a developmental period associated with heightened vulnerability to the development of ND. These results will provide the groundwork for further studies in this richly phenotyped longitudinal sample.

SUPPLEMENTARY MATERIAL

Supplementary Figures S1–S9, Table S1 and Material can be found online at http://www.ntr.oxfordjournals.org

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DECLARATION OF INTERESTS

None declared.

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