

## ORIGINAL ARTICLE

# Genetic variation in the *CHRNA5* gene affects mRNA levels and is associated with risk for alcohol dependence

JC Wang<sup>1</sup>, R Grucza<sup>1</sup>, C Cruchaga<sup>1</sup>, AL Hinrichs<sup>1</sup>, S Bertelsen<sup>1</sup>, JP Budde<sup>1</sup>, L Fox<sup>1</sup>, E Goldstein<sup>1</sup>, O Reyes<sup>1</sup>, N Saccone<sup>2</sup>, S Saccone<sup>1</sup>, X Xuei<sup>3</sup>, K Bucholz<sup>1</sup>, S Kuperman<sup>4</sup>, J Nurnberger Jr<sup>5</sup>, JP Rice<sup>1</sup>, M Schuckit<sup>6</sup>, J Tischfield<sup>7</sup>, V Hesselbrock<sup>8</sup>, B Porjesz<sup>9</sup>, HJ Edenberg<sup>3</sup>, LJ Bierut<sup>1</sup> and AM Goate<sup>1,2,10</sup>

<sup>1</sup>Department of Psychiatry, Washington University School of Medicine, Saint Louis, MO, USA; <sup>2</sup>Department of Genetics, Washington University School of Medicine, Saint Louis, MO, USA; <sup>3</sup>Department of Biochemistry and Molecular Genetics, Indiana University, Indianapolis, IN, USA; <sup>4</sup>Division of Child Psychiatry, University of Iowa, Iowa City, IA, USA; <sup>5</sup>Department of Psychiatry, Indiana University, Indianapolis, IN, USA; <sup>6</sup>Department of Psychiatry, San Diego VA Medical Center, UCSD, San Diego, CA, USA; <sup>7</sup>Department of Genetics/HGI, Rutgers University, Piscataway, NJ, USA; <sup>8</sup>Department of Psychiatry, University of Connecticut, Farmington, CT, USA; <sup>9</sup>Department of Psychiatry, SUNY Health Science Center, Brooklyn, NY, USA and <sup>10</sup>Department of Neurology, Washington University School of Medicine, Saint Louis, MO, USA

**Alcohol dependence frequently co-occurs with cigarette smoking, another common addictive behavior. Evidence from genetic studies demonstrates that alcohol dependence and smoking cluster in families and have shared genetic vulnerability. Recently a candidate gene study in nicotine dependent cases and nondependent smoking controls reported strong associations between a missense mutation (rs16969968) in exon 5 of the *CHRNA5* gene and a variant in the 3'-UTR of the *CHRNA3* gene and nicotine dependence. In this study we performed a comprehensive association analysis of the *CHRNA5-CHRNA3-CHRNA4* gene cluster in the Collaborative Study on the Genetics of Alcoholism (COGA) families to investigate the role of genetic variants in risk for alcohol dependence. Using the family-based association test, we observed that a different group of polymorphisms, spanning *CHRNA5-CHRNA3*, demonstrate association with alcohol dependence defined by *Diagnostic and Statistical Manual of Mental Disorders*, 4th edn (DSM-IV) criteria. Using logistic regression we replicated this finding in an independent case-control series from the family study of cocaine dependence. These variants show low linkage disequilibrium with the SNPs previously reported to be associated with nicotine dependence and therefore represent an independent observation. Functional studies in human brain reveal that the variants associated with alcohol dependence are also associated with altered steady-state levels of *CHRNA5* mRNA.**

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## Introduction

It is well established that alcohol and tobacco use are highly correlated in humans. Current smokers are more likely to drink heavily and to binge drink than those who have never smoked, and alcoholics smoke more heavily and endorse nicotine withdrawal symptoms at a higher rate than nonalcoholics.<sup>1–5</sup> The National Longitudinal Epidemiologic Survey reported that early onset smoking was a significant predictor of lifetime drinking and subsequent progression to lifetime alcohol abuse and dependence.<sup>6</sup> Both alcohol dependence and habitual smoking are transmitted in

families and genetic factors contribute to the development of both of these disorders.<sup>7–14</sup>

Evidence from electrophysiological, pharmacological and neurochemical studies suggest that ethanol may interact with nicotinic acetylcholine receptors (nAChR).<sup>15–18</sup> The nAChR gene family has 11 known subunits ( $\alpha_2$ ,  $\alpha_3$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\alpha_6$ ,  $\alpha_7$ ,  $\alpha_8$ ,  $\alpha_9$  and  $\beta_2$ ,  $\beta_3$ ,  $\beta_4$ ); these subunits form pentameric receptors with different combinations of subunits.<sup>19,20</sup> The effects of ethanol on nAChRs depend on the receptor subunit composition. Studies using different nAChR subtype compositions expressed in *Xenopus* oocytes demonstrate that ethanol tends to increase nicotine responsiveness in  $\alpha_2\beta_2$ ,  $\alpha_3\beta_2$  and  $\alpha_4\beta_2$  receptor subtypes, whereas low concentrations of ethanol inhibit homomeric  $\alpha_7$ -receptor function.<sup>16,21</sup> Ethanol also modulates nAChR potentiation. The combinations of  $\alpha_2\beta_4$  and  $\alpha_4\beta_2$  are the most sensitive receptors to potentiation by ethanol. The  $\alpha_4\beta_4$  and

Correspondence: Professor A Goate, Department of Psychiatry, School of Medicine, Washington University, 660 S. Euclid, Saint Louis, MO 63110, USA.

E-mail: goate@icarus.wustl.edu

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$\alpha 2\beta 2$  combinations are slightly less sensitive and the  $\alpha 3\beta 2$  and  $\alpha 3\beta 4$  combinations are insensitive to ethanol.<sup>16</sup>

Several genetic association studies involving addiction in humans have focused on the genes encoding the major nAChR subunits expressed in the brain ( $\alpha 4$  and  $\beta 2$ ). A family-based study in human populations reported genetic variants in *CHRNA4* and *CHRNA2* that are significantly associated with a protective effect against nicotine addiction.<sup>22</sup> The involvement of *CHRNA4*, but not *CHRNA2*, in nicotine addiction was confirmed in another family-based study.<sup>23</sup> A population-based study among ethnically diverse young adults reported that a polymorphism located immediately upstream of *CHRNA2* was associated with initial subjective response to both alcohol and tobacco.<sup>24</sup>

Recently a comprehensive genome-wide association study and a candidate gene study using nicotine dependent smokers as cases and nondependent smokers as controls demonstrated significant association between several genetic variants in nicotine receptors and nicotine dependence.<sup>25,26</sup> As *CHRNA5*, *CHRNA3* and *CHRNA4* genes cluster together on chromosome 15q, we performed a comprehensive association analysis with this gene cluster in the families of the Collaborative Study on the Genetics of Alcoholism (COGA) to investigate the role of genetic variants in these three nAChRs in risk for alcohol dependence. We also confirmed our findings in an independent dataset.

## Materials and methods

### Study subjects

Alcohol-dependent probands, defined by meeting lifetime criteria for both *Diagnostic and Statistical Manual of Mental Disorders*, 3rd ed (DSM-III-R) alcohol dependence<sup>27</sup> and Feighner criteria for definite alcoholism<sup>28</sup> were systematically recruited from alcohol-treatment units. Families in which two additional first-degree relatives also met lifetime criteria for alcohol dependence were invited to participate in the genetic protocol. A total of 262 families including 2309 individuals were selected for the genetic study and an average of 4.6 alcohol-dependent individuals per pedigree were genotyped.<sup>29,30</sup> ([http://www.niaaagenetics.org/coga\\_instruments/resources.html](http://www.niaaagenetics.org/coga_instruments/resources.html)). Among these pedigrees, 298 individuals from 35 pedigrees are of African-American descent and 8 pedigrees are of mixed ancestry (by self-report).

All subjects were assessed using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA).<sup>31,32</sup> Affected individuals were those who were alcohol dependent by DSM-IV criteria. Unaffected individuals were those who drank but had no more than two DSM-IV symptoms of alcohol dependence and were not dependent on any illicit substance. When multiple interviews were available, we required consistency in all interviews for affected/unaffected status.

### Genotyping assays

dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) was used to identify polymorphisms within and flanking the genes encoding *CHRNA5*, *CHRNA3* and *CHRNA4* on the long arm of chromosome 15. We used Sequenom MassArray technology (<http://www.sequenom.com>), homogenous MassEXTEND (hME) or iPLEX assays for genotyping of single nucleotide polymorphisms (SNPs). PCR primers, termination mixes and multiplexing capabilities were determined with Sequenom MassARRAY Assay Designer software v3.1.2.2. Standard procedures were used to amplify PCR products; unincorporated nucleotides were deactivated with shrimp alkaline phosphatase. A primer extension reaction was then carried out with the mass extension primer and the appropriate termination mix (hME) or terminator (iPLEX). The primer extension products were then cleaned with resin and spotted onto a silicon SpectroChip. The chip was scanned with a mass spectrometry workstation (Bruker), and the resulting genotype spectra were analyzed with the Sequenom SpectroTYPER software v3.4. Call rates greater than 90% and HWE *P*-value >0.05 were set as quality control measures. For the 22-bp insertion/deletion (indel) polymorphism (rs3841324), PCR primers (primer sequences available on request) were selected using the MacVector 6.5.3 program (Accelrys) to yield a 166-bp or 188-bp genomic fragment containing the indel. The nonsynonymous coding SNP in exon 5, rs16969968, originally identified by sequencing 40 individuals from COGA families, was genotyped using an RFLP assay with Taq $\alpha$ I restriction enzyme. Genotypes for rs3841324 and rs16969968 were detected by electrophoresis on a 2% agarose gel.

### Statistical analyses

Linkage disequilibrium (LD) between markers was computed using the program Haploview.<sup>33</sup> The family-based association test (FBAT)<sup>34,35</sup> was used to examine association between the variants and alcohol dependence, defined by DSM-IV criteria. FBAT builds on the original time domain transmission (TDT) method<sup>36</sup> in which alleles transmitted to affected offspring are compared with the expected distribution of alleles among offspring. In particular, the method puts tests of different genetic models, tests of different sampling designs, tests involving different disease phenotypes, tests with missing parents and tests of different null hypotheses all into the same framework. Similar in spirit to a classical TDT test, the approach compares the genotype distribution observed in the cases to its expected distribution under the null hypothesis, with the null hypothesis being no linkage and no association, or no association in the presence of linkage. Here, the expected distribution is derived using Mendel's law of segregation and conditioned on the sufficient statistics for any nuisance parameters under the null. Because conditioning eliminates all nuisance parameters, the technique avoids confounding due to model misspecification as well as admixture or population

stratification.<sup>37,38</sup> The data shown in Table 1 are from the standard FBAT analysis with age and gender as covariates. To correct for multiple testing we used false discovery rate (FDR) with a default threshold of 0.05.<sup>39</sup>

*Replication study with the family study of cocaine dependence data set*

*Study subjects.* Unrelated cases and matched unrelated controls within the candidate-gene study of the family study of cocaine dependence (FSCD)

were used for this study.<sup>40</sup> Cocaine dependent subjects were recruited from publicly and privately funded inpatient and outpatient chemical dependency treatment centers in the St Louis area. Eligibility requirements included meeting DSM-IV criteria for cocaine dependence, being 18 years of age or older, speaking fluent English and having a full sibling within five years of their age who was willing to participate in the family arm of the study. Control subjects were recruited through driver's license records maintained by the Missouri Family Registry at Washington University in St Louis for research

**Table 1** FBAT analysis of SNPs in the cluster of *CHRNA5-CHRNA3-CHRNA4* genes with alcohol dependence in the COGA European-American dataset

NP	Chromosome position	Allele	MAF	N <sub>f</sub>	P-value	FDR q-value
rs1979906 <sup>a</sup>	76629344	A/G	0.44	171	0.008	0.036
rs880395 <sup>a</sup>	76631411	A/G	0.43	162	0.088	0.114
rs7164030 <sup>a</sup>	76631716	A/G	0.44	149	0.113	0.129
rs905739 <sup>b</sup>	76632165	C/T	0.22	120	0.179	0.155
rs2036527 <sup>c</sup>	76638670	C/T	0.33	145	0.166	0.155
rs3841324 <sup>a</sup>	76644877	L/S	0.44	172	0.005	0.030
rs503464 <sup>b</sup>	76644951	A/T	0.22	133	0.192	0.157
rs684513 <sup>b</sup>	76645455	C/G	0.2	116	0.277	0.194
rs667282 <sup>b</sup>	76650527	C/T	0.23	136	0.352	0.229
rs17486278 <sup>c</sup>	76654537	A/C	0.32	138	0.078	0.112
rs601079 <sup>a</sup>	76656634	A/T	0.43	180	0.026	0.059
rs680244 <sup>a</sup>	76658343	A/G	0.44	175	0.003	0.030
rs621849 <sup>a</sup>	76659916	A/G	0.44	175	0.004	0.030
rs569207 <sup>b</sup>	76660174	A/G	0.23	133	0.121	0.129
rs692780 <sup>a</sup>	76663560	C/G	0.38	172	0.013	0.039
rs16969968 <sup>c</sup>	76669980	A/G	0.34	154	0.177	0.155
rs514743 <sup>a</sup>	76671282	A/T	0.38	176	0.117	0.129
rs578776 <sup>b</sup>	76675455	C/T	0.28	146	0.81	0.417
rs6495307 <sup>a</sup>	76677376	C/T	0.43	172	0.01	0.036
rs12910984 <sup>b</sup>	76678682	A/G	0.23	132	0.345	0.229
rs1051730 <sup>c</sup>	76681394	C/T	0.32	147	0.016	0.042
rs3743078 <sup>b</sup>	76681814	C/G	0.24	137	0.671	0.381
rs3743077 <sup>a</sup>	76681951	A/G	0.42	176	0.08	0.112
rs938682 <sup>b</sup>	76683602	C/T	0.23	133	0.602	0.353
rs11637630 <sup>b</sup>	76686774	A/G	0.23	132	0.228	0.166
rs7177514 <sup>b</sup>	76694461	C/G	0.24	128	0.589	0.353
rs6495308 <sup>b</sup>	76694711	C/T	0.24	132	0.87	0.417
rs8042059 <sup>b</sup>	76694914	A/C	0.23	131	0.851	0.417
rs8042374 <sup>b</sup>	76695087	A/G	0.23	121	0.432	0.271
rs3743075 <sup>a</sup>	76696507	A/G	0.39	175	0.207	0.157
rs3743073 <sup>a</sup>	76696594	A/C	0.39	175	0.199	0.157
rs1878399 <sup>a</sup>	76699058	C/G	0.43	175	0.061	0.107
rs17487223 <sup>c</sup>	76711042	C/T	0.35	144	0.065	0.107
rs950776	76713073	C/T	0.35	164	0.872	0.417
rs11636605	76715933	A/G	0.22	112	0.975	0.455
rs9920506	76718112	A/G	0.19	102	0.716	0.383
rs3813567	76721606	C/T	0.22	125	0.698	0.383
rs17487514	76740840	C/T	0.3	156	0.052	0.105
rs1996371	76743861	A/G	0.39	154	0.155	0.155

Abbreviations: COGA, Collaborative Study on the Genetics of Alcoholism; FBAT, family-based association test; FDR, false discovery rate; MAF: minor allele frequency; N<sub>f</sub>: number of informative families; SNP, single nucleotide polymorphism. The data shown here are from the standard analysis with age and gender as covariates.

<sup>a</sup>Represents SNPs that are highly correlated ( $r^2 \geq 0.7$ ) with a 22-bp indel (rs3841324) in *CHRNA5*.

<sup>b</sup>Represents SNPs that are highly correlated with rs578776 maps to the 3'-UTR of *CHRNA3*.

<sup>c</sup>Represents SNPs that are highly correlated with a missense mutation (rs16969968) in exon 5 of *CHRNA5*.

purposes. Controls were matched to cocaine dependent subjects based on age, ethnicity, gender and zip code. Control subjects were not dependent on alcohol or drugs, including nicotine, but did use at least alcohol because non-substance using individuals are considered phenotypically unknown. The project was approved by the Washington University IRB and all subjects provided informed consent. All participants completed a modified version of the SSAGA.<sup>31,32</sup>

**Genotyping assays.** Genotyping for the FSCD study was conducted by the Center for Inherited Disease Research (CIDR) using a custom SNP array on an Illumina platform. Details of genotyping procedures are available at the CIDR website (<http://www.cidr.jhmi.edu/index.html>). Additional genotyping was performed by Sequenom assays described above.

**Statistical analysis.** LD between markers was computed using the program COCAPHASE.<sup>41</sup> We used logistic regression<sup>42</sup> to examine the association between the SNPs and DSM-IV alcohol dependence. For analysis, we selected those cases who were comorbid for DSM-IV alcohol and cocaine dependence and compared them with all of the study controls. This subset included 451 unrelated individuals of European-American descent (207 alcohol-dependent cases and 244 controls) and 424 unrelated individuals of African-American descent (185 alcohol-dependent cases and 239 controls). Separate logistic regression models were run for the European and African-American subjects as well as a combined analysis that incorporated all samples and

included race as a covariate and a genotype by race interaction term. Three logistic regression models were examined for each variant to test for additive effects and evidence of dominant or recessive modes of inheritance. The additive effect of a SNP was modeled using an ordinal measure of the number of copies of the risk allele. The dominant and recessive effects of a SNP were modeled using dichotomous indicator variables. For each variant, the model with the strongest association with DSM-IV alcohol dependence, based on the adjusted odds ratio and the magnitude of the corresponding *P*-value, is reported in Table 2.

**Gene expression analyses.** Postmortem brain tissues derived from frontal cortex and cerebellum of 48 unrelated, nondemented elderly European Americans were obtained from the brain bank of the Alzheimer's Disease Research Center at Washington University in St Louis (<http://alzheimer.wustl.edu/>). We used Qiagen's DNeasy Blood & Tissue Kit and RNeasy Lipid Tissue kit (<http://www.qiagen.com>) to extract DNA and total RNA from brain tissue, respectively. A cDNA library was prepared from total RNA using the High Capacity cDNA Archive Kit (<http://www.appliedbiosystems.com>).

Genomic DNA from all subjects was genotyped for the SNP, rs588765, which exhibits the lowest *P*-value in the African-American population. The polymorphism, rs3841324 was also genotyped because of its location within the promoter of the *CHRNA5* gene. Gene expression levels were analyzed by real-time PCR using an ABI-7500 real-time PCR system. A TaqMan assay (Hs00181248\_m1, ABI) was used

**Table 2** Logistic regression analysis (with age and gender as covariates) of selected SNPs with alcohol dependence in FSCD dataset

SNP	European Americans			African Americans			Combined dataset	
	MAF	OR (CI)	P-value	MAF	OR (CI)	P-value	OR (CI)	P-value
rs880395	0.46	1.32 (1.02, 1.71)	<b>0.037</b>	0.23	1.25 (0.89, 1.76)	0.193	1.29 (1.05, 1.58)	<b>0.015</b>
rs7164030	0.47	1.35 (1.04, 1.75)	<b>0.023</b>	0.23	1.24 (0.88, 1.74)	0.221	1.30 (1.06, 1.60)	<b>0.012</b>
rs3841324	0.47	1.35 (1.04, 1.76)	<b>0.022</b>	0.22	1.24 (0.88, 1.75)	0.219	1.31 (1.07, 1.62)	<b>0.01</b>
rs588765	0.46	1.36 (1.05, 1.77)	<b>0.020</b>	0.31	1.61 (1.16, 2.23)	<b>0.004</b>	1.45 (1.19, 1.78)	<b>0.0003</b>
rs6495306	0.46	1.36 (1.05, 1.77)	<b>0.020</b>	0.31	1.55 (1.12, 2.15)	<b>0.008</b>	1.43 (1.17, 1.76)	<b>0.001</b>
rs601079	0.46	1.34 (1.03, 1.74)	<b>0.028</b>	0.43	1.37 (1.02, 1.84)	<b>0.034</b>	1.35 (1.11, 1.64)	<b>0.002</b>
rs680244	0.46	1.35 (1.04, 1.75)	<b>0.023</b>	0.43	1.36 (1.02, 1.82)	<b>0.039</b>	1.35 (1.11, 1.64)	<b>0.002</b>
rs692780	0.4	1.37 (1.05, 1.78)	<b>0.022</b>	0.24	1.47 (1.04, 2.08)	<b>0.029</b>	1.41 (1.14, 1.74)	<b>0.002</b>
rs11637635	0.40	1.33 (1.02, 1.74)	<b>0.032</b>	0.28	1.61 (1.16, 2023)	<b>0.005</b>	1.44 (1.17, 1.77)	<b>0.001</b>
rs555018	0.46	1.36 (1.05, 1.77)	<b>0.020</b>	0.31	1.58 (1.14, 2.20)	<b>0.006</b>	1.44 (1.18, 1.77)	<b>0.0005</b>
rs647041	0.46	1.33 (1.02, 1.72)	<b>0.033</b>	0.30	1.61 (1.16, 2.25)	<b>0.005</b>	1.43 (1.17, 1.75)	<b>0.001</b>
rs615470	0.40	1.29 (0.99, 1.69)	0.055	0.42	1.48 (1.10, 1.99)	<b>0.009</b>	1.38 (1.13, 1.68)	<b>0.001</b>
rs6495307	0.45	1.30 (1.00, 1.69)	<b>0.046</b>	0.42	1.47 (1.09, 1.97)	<b>0.012</b>	1.37 (1.13, 1.67)	<b>0.001</b>
rs3743077	0.46	1.34 (1.03, 1.74)	<b>0.027</b>	0.14	1.18 (0.75, 1.86)	0.471	1.38 (1.10, 1.72)	<b>0.005</b>
rs2869546	0.41	1.34 (1.03, 1.74)	<b>0.030</b>	0.39	1.18 (0.89, 1.57)	0.256	1.26 (1.04, 1.53)	<b>0.018</b>
rs3743075	0.4	1.29 (0.99, 1.68)	0.058	0.45	1.10 (0.83, 1.47)	0.511	1.20 (0.99, 1.46)	0.063
rs3743074	0.41	1.32 (1.02, 1.73)	<b>0.038</b>	0.45	1.09 (0.82, 1.45)	0.561	1.21 (1.00, 1.47)	0.053
rs1878399	0.46	1.32 (1.02, 1.71)	<b>0.038</b>	0.26	1.09 (0.79, 1.51)	0.601	1.22 (1.00, 1.50)	0.052

Abbreviations: CI, confidence interval; FSCD, family study of cocaine dependence; MAF, minor allele frequency; OR, odd ratio; SNP, single nucleotide polymorphism. *P*-values < 0.05 are in bold face.

for quantifying the expression level of *CHRNA5* in the frontal cortex. The expression levels of *CHRNA3* were determined in cerebellum using a TaqMan assay (Hs00609519, ABI) due to the low expression levels of *CHRNA3* in frontal cortex. Primers and a TaqMan probe (sequences available on request) for the reference gene, *GAPDH* were designed over exon–exon boundaries using the Primer Express 3 (ABI) program.

Each real-time PCR run included within-plate duplicates and each experiment was performed twice for each sample. Correction for sample-to-sample variation was done by simultaneously amplifying *GAPDH* as a reference. Real-time data were analyzed using the comparative  $C_t$  method.<sup>43</sup> The  $C_t$  values of each sample were normalized with the  $C_t$  value for the housekeeping gene, *GADPH* and were corrected for the PCR efficiency of each assay,<sup>43</sup> although the efficiency of all reactions was close to 100%. Only the samples with an s.e. <0.15 were analyzed. We used the nonparametric Mann–Whitney *U*-statistic to test for evidence of differential expression in samples of different genotypes.

## Results

Forty-one single nucleotide polymorphisms (SNPs) and an indel within and flanking this cluster of nAChRs were genotyped (Figure 1a). Each of the polymorphisms was in Hardy–Weinberg equilibrium in the founders. Three SNPs that had a minor allele frequency (MAF) less than 5% were removed from the analyses. Using pair-wise linkage disequilibrium analysis, we observed three groups of highly correlated variants tagged by three putative functional polymorphisms, a 22 bp indel (rs3841324) in the promoter region of the *CHRNA5* gene, a missense mutation (rs16969968) in exon 5 of the *CHRNA5* gene, and a SNP (rs578776) in the 3'-UTR of the *CHRNA3* gene, respectively (Figure 1a; Table 1).

### *Genetic variants in the CHRNA5–CHRNA3–CHRNA4 gene cluster are associated with alcohol dependence in the COGA data set*

Eight variants spanning 52 kb in the *CHRNA5–CHRNA3–CHRNA4* gene cluster were significantly associated with alcohol dependence using FBAT analysis adjusted for age and gender (Table 1). Seven of these variants are highly correlated ( $r^2 \geq 0.7$ , Figure 1b). These associated polymorphisms include rs1979906 in the region upstream of the *CHRNA5* gene, a 22 bp indel (rs3841324) in the promoter region of *CHRNA5*, four intronic SNPs in the *CHRNA5* gene and one intronic SNP in the *CHRNA3* gene (Figure 1a; Table 1). The eighth SNP (rs1051730), a synonymous variant in exon 5 of the *CHRNA3* gene has a lesser degree of linkage disequilibrium ( $r^2 \leq 0.4$ ). To determine whether this association was driven by nicotine dependence, we also analyzed the association with habitual smoking as a covariate and found that the association of these polymorphisms with alcohol dependence is independent of smoking status. These

polymorphisms remain significant after adjusting for multiple testing using the FDR.<sup>39</sup>

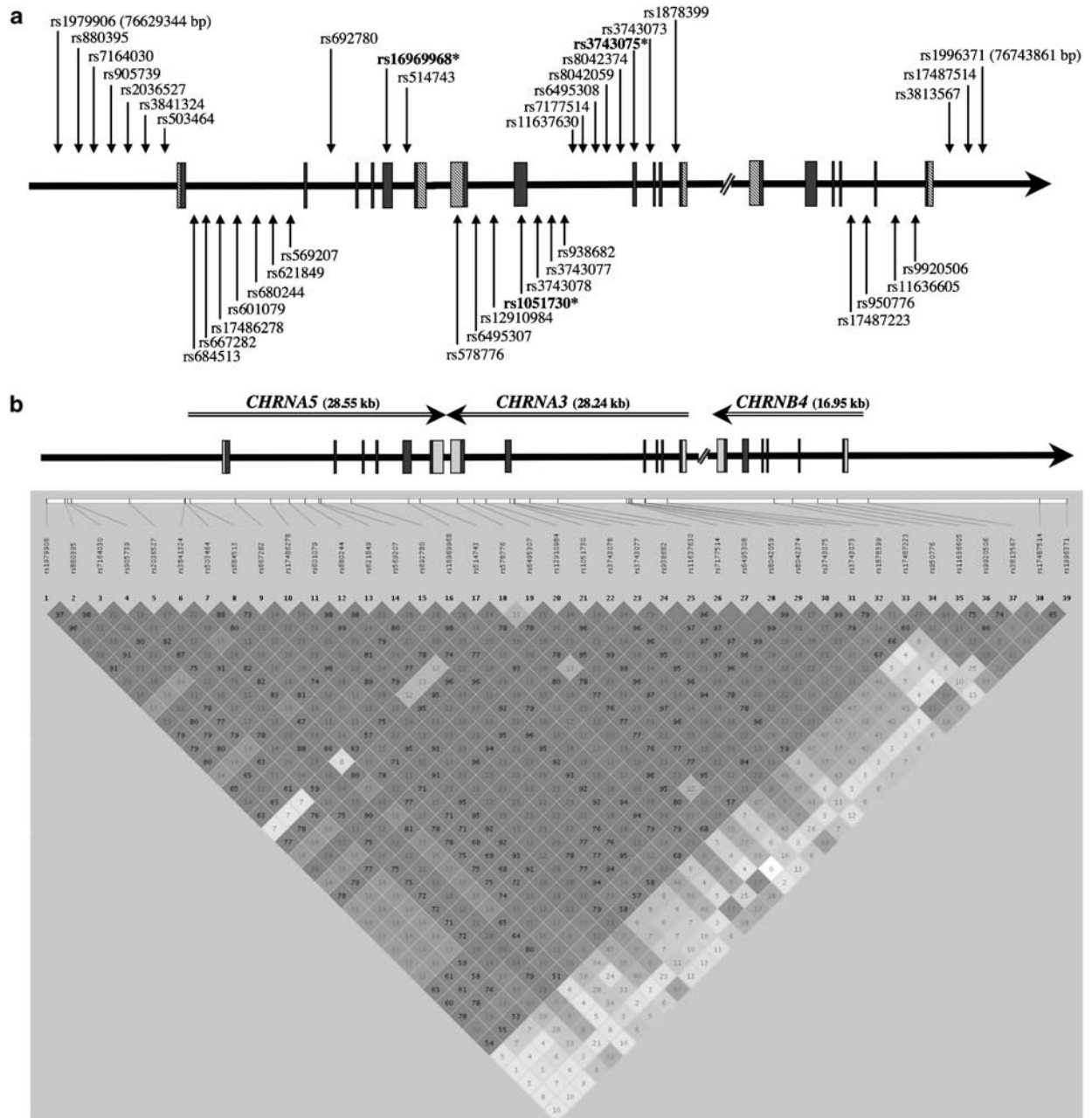
In contrast, we observed no association between alcohol dependence and either of the SNPs previously reported to be associated with nicotine dependence: the missense mutation (rs16969968) in *CHRNA5* and rs578776 located within the 3'-UTR of *CHRNA3*.<sup>26,44</sup> A similar pattern of association was seen for all polymorphisms across the gene cluster in affected only analyses and in analyses without covariates.

### *Replication of the association with alcohol dependence in an independent data set from the FSCD*

To further examine the genetic contribution of variants in this gene cluster to risk for alcohol dependence we genotyped 18 polymorphisms showing high linkage disequilibrium with each other ( $r^2 \geq 0.7$  in European Americans), in an independent data set from the FSCD (Table 2). Using logistic regression analysis we confirmed the association between each of these SNPs and alcohol dependence in the subjects of European descent. In the African-American subset, 10 of the 18 polymorphisms, spanning *CHRNA5* and *CHRNA3*, showed significant association with alcohol dependence. Six of these polymorphisms have a lower MAF in African Americans (<0.31 vs 0.4) but slightly higher odds ratios than in the European American sample (Table 2). To test whether the genetic associations observed show statistically significant differences between the two racial groups we repeated the logistic regression including a genotype by race interaction term. None of the variants showed significant evidence of genetic heterogeneity by race.

### *Allelic differences in expression of CHRNA5 in human frontal cortex*

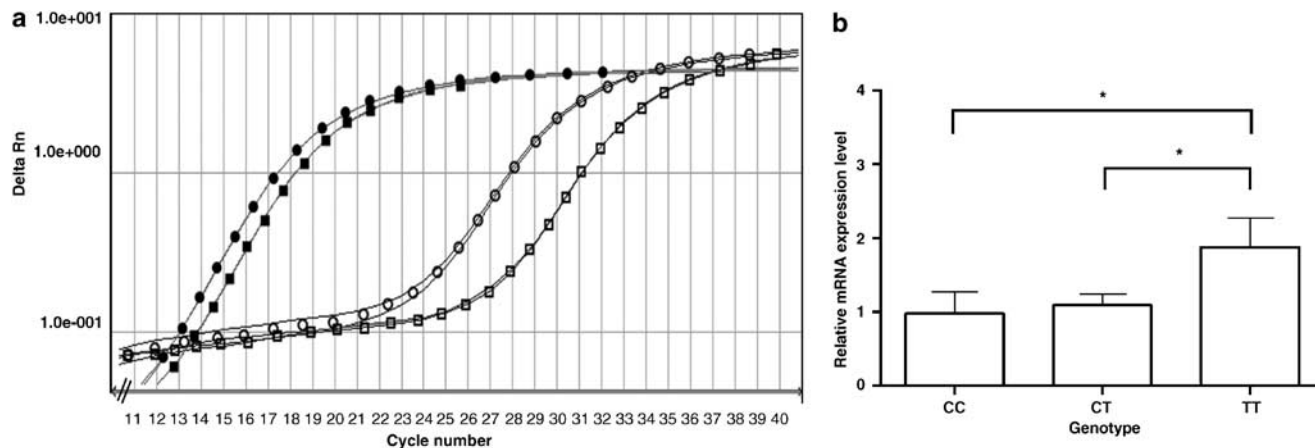
To examine whether the variants associated with alcohol dependence have a direct effect on gene expression, we performed quantitative real-time PCR analysis with human brain tissue from nondemented elderly European Americans. Genomic DNA from all subjects was genotyped for multiple variants in *CHRNA5*. Genotypes were in Hardy–Weinberg equilibrium. We then examined *CHRNA5* mRNA expression with SNP, rs588765 in 13 samples homozygous for the major allele (CC), 9 samples homozygous for the minor allele (TT), and 21 heterozygous samples (CT). Subjects homozygous for the minor allele of rs588765 showed a 2-fold increase ( $TT = 1.88 \pm 1.2$ ;  $CC = 0.98 \pm 1.04$ ;  $P = 0.025$ ) in *CHRNA5* mRNA expression compared to subjects homozygous for the major allele (Figure 2). Similar results were obtained with rs3841324 ( $SS = 2.21 \pm 1.15$ ;  $LL = 0.89 \pm 0.89$ ;  $P = 0.02$ ). Heterozygotes for both rs588765 and rs3841324 showed lower expression levels for *CHRNA5*, compared with homozygotes for the minor allele ( $P = 0.0003$  and  $P = 0.02$  for rs3841324 and rs588765, respectively). However, no significant differences were found between heterozygotes and homozygotes



**Figure 1** (a) Location of genotyped polymorphisms across the cluster of *CHRNA5-CHRNA3-CHRNA4* genes. The dark boxes represent exons. The hatched boxes represent 5'- and 3'-UTRs; \* represents coding single nucleotide polymorphisms (SNPs). Diagram is not drawn to scale. (b) Pair-wise linkage disequilibrium among polymorphisms in the region of *CHRNA5-CHRNA3-CHRNA4* genes in COGA European-American data set. Numbers in boxes represent  $r^2$  value between variants.

for the major allele (Figure 2). In contrast *CHRNA3* expression in the same individuals showed no association with alleles of rs588765 or rs3841324 (data not shown). This expression data suggest that the functional allele explaining the association with alcohol dependence regulates *CHRNA5* expression. However, in these European American samples available for our expression studies we are not able to determine which variant is responsible for the expression differences.

To validate this observation using an independent data set and methodology we tested for association between variability in *CHRNA5* mRNA expression in lymphoblastoid cell lines derived from CEPH families using the Affymetrix HG Focus panel (Genetic Analysis Workshop 15; [www.gaworkshop.org/gaw15.htm](http://www.gaworkshop.org/gaw15.htm)).<sup>45,46</sup> We retrieved genotypes for rs588765 from the HapMap database and examined the difference in *CHRNA5* mRNA expression in subjects with different genotypes at rs588765 in 14 genotyped trios. Using



**Figure 2** Association of the single nucleotide polymorphism (SNP), 588765 with *CHRNA5* mRNA expression. (a) The  $\Delta Rn$  vs cycle plot showing relative total expression of *CHRNA5* (open circles and open squares) and *GAPDH* (solid circles and solid squares). Circles represent a sample homozygous for minor allele (TT) and squares represent a sample homozygous for major allele (CC). The level of expression was calculated from the  $C_t$  value (the cycle at which the fluorescence intensity rises above a threshold) and was normalized by taking *GAPDH* as a reference. (b) Mann–Whitney *U*-statistic, two-tailed analysis of *CHRNA5* total expression in subjects with homozygous for the major allele (CC), subjects with homozygous for the minor allele (TT) and heterozygous subjects (CT). y-Axis represents the relative expression level taking an arbitrary reference samples as 1. Mean  $\pm$  s.d. is shown; \* indicates *P*-value  $< 0.05$ .

SOLAR VC quantitative analysis of the *CHRNA5* mRNA levels with additive genetic effects, we detected significant differences in expression in subjects of different genotypes ( $P = 0.02$ ). This SNP accounted for approximately 14% of the variance in *CHRNA5* gene expression in this system.

## Discussion

Alcohol dependence frequently co-occurs with tobacco use. Evidence from electrophysiological and neurochemical studies suggest that ethanol and nicotine share important mechanisms of action in the brain reward pathways and nAChRs.<sup>17</sup> A study using the COGA data set demonstrated that the clinical characteristics of alcohol use history are associated with smoking status; current smokers and nicotine-dependent subjects had a greater severity of alcohol dependence.<sup>3</sup>

Although the main nAChRs in the brain are believed to contain  $\alpha 4$  and  $\beta 2$  subunits, a recent comprehensive candidate gene study implicated SNPs in the genes encoding the  $\alpha 3$ ,  $\alpha 5$ , and  $\beta 3$  subunits, including a missense mutation, rs16969968, in the *CHRNA5* gene as risk factors for nicotine dependence.<sup>26</sup> Given the shared genetic vulnerability for nicotine and alcohol dependence, detected through twin studies,<sup>9,14</sup> we undertook a detailed analysis of the *CHRNA5–CHRNA3–CHRNA4* gene cluster in the COGA data set. Although the association between the missense mutation in *CHRNA5* and nicotine dependence has been replicated in several data sets, including the COGA data set,<sup>44</sup> we saw no evidence of association between this SNP and alcohol dependence, suggesting that the effect of this polymorphism is specific to nicotine. Similarly, the group

of SNPs in linkage disequilibrium with rs578776 shows strong association with nicotine dependence<sup>26</sup> but shows no evidence of association with alcohol dependence in the COGA data set.

In this study we provide replicated evidence of association for 16 correlated variants with alcohol dependence in two data sets of European descent. The strong association detected in the FSCD data set, where all cases are both cocaine dependent and alcohol dependent, raises the possibility that these variants may also be associated with alcohol related phenotypes, such as illicit drug dependence and antisocial personality disorder. With lower levels of LD among the polymorphisms in the African-American population (Table 3), we observed a narrower region of association extending from rs588765 in intron 1 of *CHRNA5* to rs6495307 in intron 5 of *CHRNA3*. The SNPs showing the lowest *P*-values in the African-American sample have a MAF of 30% compared to 46% in the European-American sample. As we do not see genetic association with the indel in African Americans, this suggests that further studies should prioritize SNPs in high LD with rs588765. Although there are differences in the LD between the polymorphisms in the European-American and African-American subjects from the FSCD data set, none of these variants demonstrated significant evidence of genetic heterogeneity between the two populations. Thus, though there are some differences in significance and effect sizes between the variants, we cannot definitively determine which polymorphism is causing the functional changes.

Using brain mRNA from European Americans we observed an association between the minor alleles of rs588765 and rs3841324 ( $r^2 = 0.8$  in European Americans) and higher levels of *CHRNA5* mRNA.

**Table 3** Pair-wise linkage disequilibrium among single nucleotide polymorphisms (SNPs) in family study of cocaine dependence (FSCD) African-American dataset

	rs880395	rs7164030	rs3841324	rs6495306	rs601079	rs680244	rs692780	rs11637635	rs555018	rs647041	rs615470	rs6495307	rs3743077	rs2869546	rs3743074	rs1878399
rs880395	1.00															
rs7164030	0.39	1.00														
rs3841324	0.39	0.39	1.00													
rs588765	0.13	0.13	0.13	1.00												
rs6495306	0.68	0.68	0.10	0.68	1.00											
rs601079	0.67	0.67	0.09	0.54	0.97	1.00										
rs680244	0.38	0.38	0.02	0.56	1.00	0.56	1.00									
rs692780	0.86	0.86	0.10	0.77	0.42	0.42	0.42	1.00								
rs11637635	0.70	0.70	0.07	0.91	0.52	0.52	0.82	0.82	1.00							
rs555018	0.67	0.67	0.09	1.00	0.56	0.56	0.76	0.76	1.00	1.00						
rs647041	0.71	0.71	0.09	0.95	0.54	0.54	0.79	0.79	1.00	1.00	1.00					
rs615470	0.33	0.33	0.01	0.50	0.13	0.14	0.40	0.49	0.50	0.48	0.48	1.00				
rs6495307	0.34	0.34	0.02	0.52	0.15	0.16	0.38	0.47	0.51	0.49	0.85	0.85	1.00			
rs3743077	0.38	0.38	0.02	0.38	0.21	0.21	0.45	0.38	0.38	0.40	0.84	0.84	0.83	1.00		
rs2869546	0.34	0.34	0.02	0.38	0.23	0.23	0.26	0.24	0.24	0.26	0.82	0.82	0.80	0.80	1.00	
rs3743075	0.27	0.27	0.24	0.19	0.02	0.02	0.30	0.21	0.18	0.18	0.50	0.50	0.77	0.77	1.00	
rs3743074	0.27	0.27	0.24	0.18	0.02	0.02	0.30	0.20	0.17	0.17	0.98	0.48	0.76	0.76	1.00	
rs1878399	0.67	0.67	0.07	0.80	0.45	0.45	0.73	0.57	0.79	0.83	0.83	0.40	0.47	0.47	0.38	0.37

This association with *CHRNA5* expression was replicated in the GAW15 data set with rs588765.<sup>45,46</sup> Several in-vitro studies have examined 5' regulatory sequences of *CHRNA5*.<sup>47-49</sup> Functional characterization of *CHRNA5* using luciferase assays in human cell lines has previously demonstrated that the -240/+53 region, which contains the rs3841324 indel, is the core promoter.<sup>47,48</sup> A second study has directly tested the effect of the indel using a standardized reporter gene assay system and reported that it alters promoter activity in HEK293t cells by 1.5-fold.<sup>49</sup> However, our genetic data in African Americans does not support a primary role of this polymorphism in influencing *CHRNA5* expression in the brain. No in-vitro functional experiments have been performed using other putative regulatory regions of *CHRNA5*. Further examination on *CHRNA5* mRNA expression in African-American subjects may be very helpful in narrowing down the likely functional variant because of the lower levels of LD across *CHRNA5* in this population.

In the mammalian brain, nAChRs include homopentameric  $\alpha7$  receptors and a variety of heteropentamers, but predominantly  $\alpha4\beta2^*$ , where the asterisk denotes the presence of another subunit. In many brain areas, the  $\alpha5$  polypeptide completes the  $\alpha4\beta2^*$  complex, with stoichiometry  $\alpha4_2\beta2_2\alpha5$ .<sup>20,50,51</sup> The  $\alpha5$  subunit contributes one-fifth of the channel lining and therefore can influence channel properties.<sup>20</sup> Studies using heterologous expression systems have demonstrated that addition of the  $\alpha5$  subunit has profound effects on the functional properties of  $\alpha3\beta4$  receptors.<sup>52</sup> *CHRNA5* increases the rate of desensitization of  $\alpha3\beta4$  receptors as well as the Ca<sup>2+</sup> permeability and decreases the efficacy of nicotine on  $\alpha3\beta4$  nAChRs.

In conclusion, we provide replicated evidence of association between multiple polymorphisms within the *CHRNA5* and *CHRNA3* genes and alcohol dependence. Furthermore, we demonstrate that the minor alleles of these polymorphisms are associated with higher *CHRNA5* mRNA levels in human frontal cortex. These results suggest that although variation in *CHRNA5* influences risk for both alcohol dependence and nicotine dependence, different polymorphisms and different mechanisms of action are responsible for these effects on risk.

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