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Genome-wide meta-analysis identifies a novel susceptibility signal at *CACNA2D3* for nicotine dependence

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Abstract

Nicotine dependence (ND) has a reported heritability of 40–70%. Low-coverage whole-genome sequencing was conducted in 1,889 samples from the UCSF Family study. Linear mixed models were used to conduct genome-wide association tests of ND in this and five cohorts obtained from the database of Genotypes and Phenotypes. Fixed-effect meta-analysis was carried out separately for European ($n = 14,713$) and African ($n = 3,369$) participants, and then in a combined analysis of both ancestral groups. The meta-analysis of African participants identified a significant and novel susceptibility signal (rs56247223; $P = 4.11 \times 10^{-8}$). Data from the Genotype-Tissue Expression (GTEx) study suggested the protective allele is associated with reduced mRNA expression of *CACNA2D3* in three human brain tissues ($P < 4.94 \times 10^{-2}$). Sequence data from the UCSF Family study suggested that a rare nonsynonymous variant in this gene conferred increased risk for ND ($P = 0.01$) providing further support for *CACNA2D3* involvement in ND. Suggestive associations were observed in six additional regions in both European and merged populations ($P < 5.00 \times 10^{-6}$). The top variants were found to regulate mRNA expression levels of genes in human brains using GTEx data ($P < 0.05$): *HAX1* and *CHRNA2* (rs1760803), *ADAMTSL1* (rs17198023), *PEX2* (rs12680810), *GLIS3* (rs12348139), non-coding RNA for *LINC00476* (rs10759883), and *GABBR1* (rs56020557 and rs62392942). A gene-based association test further supported the relation between *GABBR1* and ND ($P = 6.36 \times 10^{-7}$). These findings will inform the biological mechanisms and development of therapeutic targets for ND.

Keywords

Nicotine dependence; genome-wide meta-analysis; susceptibility genes; expression quantitative trait locus; nonsynonymous variants

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Introduction

Tobacco usage is the leading cause of preventable mortality worldwide. Vaporized inhaled nicotine from combusted tobacco is an efficient delivery system that frequently leads to nicotine dependence (ND) [Sullivan and Kendler 1999]. The lifetime incidence of ND may be as high as 25% and is similar across diverse populations [Breslau et al., 2001]. ND clusters in families, and large twin studies indicate that ND has a moderate genetic predisposition, with an estimated heritability between 40% and 70% [Kendler et al., 1999; Sullivan and Kendler 1999; Vink et al., 2005].

Over the past few decades, many large-scale genome-wide association (GWA) studies and meta-analyses have identified a number of single nucleotide polymorphisms (SNPs) associated with the risk of ND and smoking-related behaviors [Bierut et al., 2007; David et al., 2012; Gelernter et al., 2015; Gizer and Ehlers 2015; Hancock et al., 2015; Liu et al., 2010; Loukola et al., 2014; Nishizawa et al., 2015; Rice et al., 2012; Thorgeirsson et al., 2008; Thorgeirsson et al., 2010; Tobacco and Genetics Consortium 2010; Uhl et al., 2008; Zuo et al., 2013]. Among them, the $\alpha 3/\alpha 5/\beta 4$ cholinergic nicotinic receptor subunit gene cluster on chromosome 15 consistently shows the most significant replicable risk effect on ND, smoking cessation, smoking quantity, and other smoking-related traits [Thorgeirsson et al., 2008; Thorgeirsson et al., 2010]. In 2015, Gelernter et al. conducted a genome-wide meta-analysis on ND, as measured by the Fagerström Test for ND score. They found that more GWA signals were identified in African American than in European American samples, highlighting the benefit of analyzing populations with different genetic backgrounds [Gelernter et al., 2015].

GWA studies can detect common genetic variants (defined as minor allele frequency [MAF] 1%). These studies have advanced our understanding of the genetics of ND and smoking-related traits, but the identified variants collectively explain only a small fraction of the total heritability of ND. It has been suggested that common variants, which typically exhibit small effect sizes (e.g. $R^2 < 0.005$), are unlikely to be identified using the customary GWA significance threshold and the sample size typical in most studies [Manolio et al., 2009]. Genome-wide meta-analysis, which is used to aggregate individual studies, is expected to improve the statistical power for associations and has been successfully used to identify novel genetic variants not previously discovered by single studies [Cho et al., 2012; David et al., 2012; Liu et al., 2010; Mahajan et al., 2014; Nalls et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium 2014]. In addition, in the context of mining existing GWA results, the incorporation of functional data in GWA studies and gene-based analysis has the potential to accelerate the discovery of more functionally relevant genes and could also help to elucidate the biological implications of GWA findings [Ioannidis et al., 2009]. Besides common variants, rare and low-frequency variants that have not been fully covered in GWA studies could explain, at least in part, the risk for common diseases/traits [Manolio et al., 2009]. Recently, multiple low-frequency and even rare coding variants have been revealed to be associated with the risk of ND in population-based studies [Doyle et al., 2014; Haller et al., 2012; Olfson et al., 2016; Thorsteinsdottir et al., 2014; Xie et al., 2011; Yang et al., 2015; Zuo et al., 2016], but the role of rare variants in the risk of ND has not been systematically investigated at the genome-wide level.

In the present study, we sought to identify novel common susceptibility SNPs partially responsible for the risk of ND through genome-wide meta-analysis, and explore their possible mechanisms in the pathogenesis of ND. Genome-wide mixed linear model (MLM)-based association analysis on ND was performed for six studies, with subjects stratified into genetically homogeneous European (EUR) and African (AFR) ancestry subgroups. Meta-analyses were carried out in the EUR, AFR, and trans-population merged cohort, which consisted of 18,082 subjects in total. We explored the regulatory effects of the identified SNPs in human brains through expression quantitative trait locus (eQTL) analysis in the Genotype-Tissue Expression (GTEx) data set, and searched for rare nonsynonymous variants in the implicated genes and several previously identified genes in the whole-genome sequencing data in the UCSF Family Alcoholism study.

Materials and Methods

UCSF Family Alcoholism Study Subjects

The UCSF Family Alcoholism study was designed to identify genetic loci that influence susceptibility to alcohol dependence and related phenotypes. It includes small nuclear families and unrelated subjects, and the majority of study subjects are EUR [Vieten et al., 2004]. A total of 2,154 individuals from 970 families from December 1995 through January 2003 were enrolled in this study. The recruitment of subjects has been described previously [Vieten et al., 2004]. Briefly, study probands from the community were recruited through direct mail, press releases, advertisements, and etc. Their relatives were invited by mail to participate. The diagnosis for ND was determined according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV). The samples in the UCSF Family Alcoholism study were subjected to low-pass whole-genome sequencing. Genotypes were imputed from the low-pass sequencing data as previously described [Bizon et al., 2014]. Briefly, low-coverage, paired-end read whole-genome sequencing for 1,889 samples was performed on Illumina HiSeq2000 sequencers. Reads from whole-genome sequencing were aligned using BWA version 0.5.8c [Li and Durbin 2010]. Linkage disequilibrium (LD)-aware variant calls were calculated in a multistage process that initially creates genotype-likelihood files for single samples using samtools-hybrid, then builds initial haplotypes using BEAGLE [Browning and Browning 2007], and then runs Thunder [Li et al., 2011] using the BEAGLE haplotypes as input [Bizon et al., 2014]. The mean coverage of the cohort was 4.2X. We applied the following quality control (QC) to the whole-genome sequencing data (MAF $\geq 1\%$, Hardy-Weinberg equilibrium (HWE) in controls $P > 1.00 \times 10^{-6}$) and included 8,893,347 common SNPs in the single-variant association test. After selection for subjects with diagnostic data and completion of QC steps, 918 subjects were identified as ND cases and 921 were free of ND (Supplementary Table I).

Database of Genotypes and Phenotypes (dbGaP) Subjects

We accessed five GWA data sets (SAGE: Study of Addiction: Genetics and Environment; GASSC: The Genetic Architecture of Smoking and Smoking Cessation; ADGE: Alcohol Dependence GWAS in European and African Americans; ICGHD: International Consortium on the Genetics of Heroin Dependence; OZALC: Alcohol Research using Australian twins and their families) through dbGaP [Bierut et al., 2010; Bierut et al., 2008b; Cornelis et al.,

2010; Hinrichs et al., 2006; Shand et al., 2010; Xie et al., 2011]. The ICGHD data set consisted of three subsets (CIDR: Center for Inherited Disease Research; RATRP: Research on Alcohol and Tobacco Related Phenotypes; and ARC: Alcohol Related Conditions) that were genotyped using three different arrays. The sample sizes ranged from 469 to 6,695 individuals. All subjects were recruited for genetic studies of substance use disorders. The DSM-IV criteria for ND were used as a measure of ND for all studies. The procedures for data collection for these cohorts were described in detail in dbGaP (<http://www.ncbi.nlm.nih.gov/gap>) and in previous literature [Bierut et al., 2008a; Edenberg 2002; Gelernter et al., 2007; Lessov et al., 2004]. The cohorts contained data for small families and unrelated individuals. See Table I for study characteristics and dbGaP accession numbers.

In order to facilitate the meta-analysis in more specific ancestral populations, we employed principal component analysis (PCA) to identify genetically homogeneous populations [Price et al., 2006]. In each dbGaP data set, all samples were analyzed together with the reference samples from the International HapMap Project [International HapMap Consortium 2003]. Two subsets of genetically homogeneous subjects, described here as EUR and AFR in each study, were selected using the first two principal components. The PCA plots are shown in Supplementary Figure 1.

Genomic Imputation

We applied QC filters on each individual dbGaP GWA data set (sex check, mean call rate 0.90, MAF $\geq 1\%$, HWE in controls $P > 1.00 \times 10^{-6}$). Then, whole-genome imputation was performed using the IMPUTE version 2 [Howie et al., 2012] and the integrated haplotypes of the 1000 Genomes Project Phase III reference panel (October 2014 version) [Auton et al., 2015]. Imputation was carried out in each of the seven dbGaP GWA data sets/subsets individually. We applied QC filters on the post-imputed data sets: SNPs with imputation information $< 30\%$, MAF $< 1\%$ or HWE P-value in controls $< 1.00 \times 10^{-6}$ were excluded from further analysis. Only bi-allelic autosomal SNPs were analyzed in this study.

Association and meta-analysis

The single-variant association between each common variant and the risk of ND was measured using a MLM or MLM leaving-one-chromosome-out (LOCO)-based association as implemented in GCTA for each dbGaP GWA data set and the UCSF Family Alcoholism study [Yang et al., 2011; Yang et al., 2014]. Based on previous reports, the MLM-based association appropriately corrects for population stratification and sample relatedness [Yang et al., 2014].

We performed an inverse-variance fixed-effects meta-analysis based on the cohort effect sizes and standard errors using METAL, with a Cochran's Q test to assess between-study heterogeneity [Willer et al., 2010]. Meta-analysis was performed in the 14,713 EUR, 3,369 AFR, and trans-ancestry merged cohorts (Table I). The meta-analysis for the trans-ancestry cohort aggregated ancestry-specific association result from each study. A P-value of $< 5.00 \times 10^{-8}$ was used as the threshold for genome-wide significance [Panagiotou and Ioannidis 2012].

eQTL analysis

We obtained the genotype and transcript expression data for GTEx from the dbGaP by accession number phs000424.v6.p1 [GTEx Consortium 2013]. We included only 114 subjects genotyped by the Illumina HumanOmni2.5 array. The transcript expression level was measured by RNAseq in the unit of Reads Per Kilobase of transcript per Million mapped reads (RPKM) [GTEx Consortium 2013]. Imputation was executed using IMPUTE version 2 and the 1000 Genomes Project Phase III reference panel (October 2014 version) [Auton et al., 2015; Howie et al., 2012]. Analysis of Variance was employed to evaluate the eQTL effect between the SNPs implicated in the meta-analysis and the mRNA expression level of their respective close genes in thirteen brain tissue samples (cortex, nucleus accumbens basal ganglia, amygdala, anterior cingulate cortex, caudate basal ganglia, cerebellum, frontal cortex, cerebellar hemisphere, hippocampus, hypothalamus, substantia nigra, putamen basal ganglia, and spinal cord cervical). The gender, age, and subject race for each tissue donor were included as covariates. The analysis was implemented in R 3.2.2.

Gene-based association test

We implemented a gene-based association analysis through MetaXcan using the summary statistics from the ND meta-analysis in the merged trans-population data set [Barbeira et al., 2016]. Transcript expression-level predictive models in multiple tissues were previously generated in PrediXcan using a subset of genotype and expression data from the GTEx data set. Models for ten brain tissue samples were available when the current study was conducted (cortex, putamen basal ganglia, caudate basal ganglia, frontal cortex, cerebellum, anterior cortex, hypothalamus, cerebellar hemisphere, hippocampus, and nucleus accumbens basal ganglia) [Gamazon et al., 2015]. MetaXcan uses established predictive models and accepted association summary statistics as input; it then imputes the transcript expression levels and tests the association between these imputed transcript expression levels and the phenotype [Barbeira et al., 2016]. The mathematics for the gene-based test has been described previously [Barbeira et al., 2016; Gamazon et al., 2015]. Between 8,530 and 13,570 gene transcripts were predicted with high confidence in the ten predictive models. We used as inputs for MetaXcan the ND meta-analysis association result in the trans-population merged cohort for 12,768,612 common SNPs that appeared in at least one single data set. The gene association significance threshold was set at 3.68×10^{-6} after Bonferroni correction.

Rare-variant association and gene-based test

We looked for an increased frequency of rare nonsynonymous variants in those genes implicated by the present meta-analysis (*CACNA2D3*, *GABBR1*, *CHRNA2*, *PEX2*, *ADAMTSL1*, and *GLIS3*) and several previously well-known ND susceptibility genes (*CHRNA3*, *CHRNA4*, *CHRNA5*, *CHRNA6*, *DISC1*, *DLC1*, *KLHL28*, and *C14ORF28*) [Gelernter et al., 2015; Haller et al., 2012; Hancock et al., 2015; Olfson et al., 2016; Rice et al., 2012; Thorgeirsson et al., 2010; Thorsteinsdottir et al., 2014; Xie et al., 2011] using low-pass whole-genome sequencing data in the UCSF Family Alcoholism study. A quantitative phenotype was created by regressing out the covariates of age and gender. This pseudo-quantitative trait was used for single-variant association analysis using Efficient Mixed-

Model Association eXpedited (EMMAX) in the Efficient and Parallelizable Association Container Toolbox (EPACTS) package version 3.2.6 [Kang et al., 2010]. In addition, we employed the Sequence Kernel Association Test (SKAT) under the framework of the EMMAX model in the EPACTS package to perform the gene-based test [Kang et al., 2010; Wu et al., 2011]. The gene-based test included only nonsynonymous variants (including nonsynonymous, splicing, and stop-gain variants) with allele frequency $\geq 1\%$ and allele count in the cohort of ≥ 2 . We used a significance threshold of $P < 0.05$ for single rare-variant association and gene-based tests of the described rare variants.

Results

We performed a single-variant association analysis of common variation using MLM on each individual dbGaP GWA data set and the UCSF Family Alcoholism study for the EUR and AFR samples. The quantile-quantile (Q-Q) plots for each study show that the MLM method controlled the potential inflation in each of the cohorts (Supplementary Figure 2). Fixed-effect meta-analysis was performed for 2,976,328, 4,975,009, and 2,590,787 common study-shared SNPs in the EUR, AFR, and merged cohorts, respectively. The EUR, AFR and merged cohorts consisted of 14,713, 3,369, and 18,082 samples, respectively. The Q-Q and Manhattan plots indicate that the population substructure was appropriately controlled in meta-analyses (Supplementary Figures 3–4). Although this was not an independent replication effort given the overlap of study samples with the previously published reports [Gelernter et al., 2015; Hancock et al., 2015], this meta-analyses confirmed rs1051730 in the *CHRNA5-CHRNA3-CHRNA4* region and nine other recently reported SNPs in five distinct genomic regions associated with ND with nominal association evidence ($P < 0.05$, Supplementary Table II).

Meta-analysis in the AFR cohort

In the AFR samples, SNP rs56247223 on chromosome 3 achieved a genome-wide significant association with ND (OR = 0.93, $P = 4.11 \times 10^{-8}$, MAF = 32%; Figure 1a and Supplementary Tables III–IV). SNP rs56247223 is located in an intron of the gene *CACNA2D3* (Figure 1b). The minor A allele of rs56247223 showed a modest, but consistent protective effect in the three AFR data sets (Figure 1a). The analysis of the GTEx data suggested that the minor protective A allele of SNP rs56247223 was associated with reduced levels of *CACNA2D3* mRNA in human brain cortex, frontal cortex, and putamen ($P = 5.53 \times 10^{-3}$, 4.58×10^{-2} , 4.94×10^{-2} , Figure 1c and Supplementary Figure 7).

Meta-analysis in the EUR cohort

SNP rs56247223 did not show any evidence of association with ND in the EUR cohort ($P = 0.68$, Figure 1a). No SNPs exceeded the genome-wide significance threshold in the EUR meta-analysis. However, three SNPs, rs1760803, rs17198023, and rs56020557 on chromosomes 1, 6, and 9, respectively, showed moderate and consistent association evidence with ND across samples ($P < 5.00 \times 10^{-6}$, Supplementary Figures 5–6). Among these SNPs, the most significant rs1760803 (OR = 0.97, MAF = 46%, $P = 4.26 \times 10^{-6}$), which resides in the upstream of genes *HAX1* and *CHRNA2*, exhibited a significant eQTL effect on the *HAX1* and *CHRNA2* genes in human brain cerebellum, hemisphere and hippocampus ($P =$

3.66×10^{-2} , 1.54×10^{-2} , and 2.01×10^{-2} , respectively, Supplementary Figure 7). The A allele of SNP rs17198023 exhibited considerable association with increased risk for ND in the EUR samples (OR = 1.04, MAF = 13%, $P = 4.69 \times 10^{-6}$), and was correlated with downregulation of the *ADAMTSL1* gene mRNA in human brain caudate basal ganglia ($P = 3.34 \times 10^{-2}$, Supplementary Figure 7). Notably, the third SNP, rs56020557 (OR = 1.05, MAF = 6%, $P = 4.69 \times 10^{-6}$), is 5kb upstream of the *GABBR1* gene (Supplementary Figures 5–6).

Meta-analysis in the merged cohort

In the merged EUR and AFR meta-analysis, it was noted that three SNPs in high LD (rs12680810, rs56225501, and rs28534373) located on chromosome 8 achieved moderate association evidence ($P < 2.00 \times 10^{-6}$). An examination of the study specific results indicated more consistent results for the EUR than the AFR samples (Supplementary Table V, Supplementary Figures 5–6). Among them, the most significant SNP, rs12680810 (OR = 1.03, MAF = 41% and 22% in the EUR and AFR samples, respectively, $P = 9.76 \times 10^{-7}$), showed a significant eQTL effect on the *PEX2* gene in human brain frontal cortex ($P = 1.81 \times 10^{-2}$, Supplementary Figure 7). Two additional SNPs on chromosome 9 (rs12348139 and rs10759883) were moderately associated with ND in the merged analysis ($P < 2.00 \times 10^{-6}$, Supplementary Table V, Supplementary Figures 5–6); both showed a consistent effect on the risk of ND across all individual studies and achieved at least nominal association evidence in both EUR and AFR ancestry-specific meta-analyses (Supplementary Table V, Supplementary Figures 5–6). rs12348139 is located in an intron of the *GLIS3* gene (OR = 1.04, MAF = 7% and 10% in EUR and AFR samples, respectively, $P = 1.09 \times 10^{-7}$); its minor C allele, which was associated with an increased risk for ND, upregulated the expression levels of *GLIS3* gene mRNA in human brain putamen ($P = 1.98 \times 10^{-2}$, Supplementary Figure 7). The C allele of rs10759883, located in a long non-coding RNA (*LINC00476*), was associated with increased risk of ND (OR = 0.98, MAF = 43% and 34% in the EUR and AFR samples, respectively, $P = 1.23 \times 10^{-6}$), and was highly correlated with upregulation of *LINC00476* expression levels in nine human brain regions (cortex, frontal cortex, anterior cingulate cortex, caudate basal ganglia, cerebellum, hypothalamus, substantia nigra, putamen and nucleus accumbens basal ganglia, Supplementary Figure 7). The most significant eQTL effect was observed in human brain anterior cingulate cortex ($P = 1.95 \times 10^{-5}$, Supplementary Figure 7). Finally, SNP rs62392942 achieved significant association and showed identical effect direction in the EUR and AFR samples, although it achieved nominal significance only in the EUR meta-analysis (Supplementary Table V and Supplementary Figure 6). SNP rs62392942 resides downstream of the *GABBR1* gene on chromosome 6, and is highly correlated with rs56020557 among EUR individuals (LD: $r^2 = 0.86$ and 0.48 in EUR and AFR samples, respectively). Notably, rs56020557 was among the top signals in the EUR meta-analysis ($P = 4.50 \times 10^{-6}$). The C allele of rs62392942, which was associated with increased risk for ND, exhibited an eQTL effect on *GABBR1* gene mRNA in human brain putamen (Supplementary Figure 7). Using the merged EUR and AFR meta-analysis summary statistics, the *GABBR1* gene was significantly associated with risk for ND in the gene-based MetaXcan analysis ($P = 6.36 \times 10^{-7}$).

Rare variants and gene-based tests

In the UCSF Family Alcoholism study, we performed exploratory single rare-variant association and gene-based tests with the aim of exploring the possible mechanism(s) underlying the associations between the implicated genes, as well as several previously established ND genes. We observed a risk overrepresentation of rare nonsynonymous variants among ND cases relative to controls in the *HAX1* gene (gene-based test $P = 0.03$, Table II). Moreover, a rare nonsynonymous variant in the *HAX1* gene conferred a nominally significant protective effect on ND (chr1:154247908:C/T, MAF = 0.14%, OR = 0.31, $P = 2.80 \times 10^{-2}$, Supplementary Table VI). In contrast, the gene-based test of rare nonsynonymous variants in the *CACNA2D3* gene was not significant ($P = 0.77$), though a rare nonsynonymous variant c.C1604T in exon 17 of *CACNA2D3* was nominally associated with ND (MAF = 0.16%, OR = 3.66, $P = 1.10 \times 10^{-2}$, Supplementary Table VI). Six heterozygotes subjects were identified with this rare variant, and they were all diagnosed with ND. Similarly, although no aggregate effect of rare nonsynonymous variants was discovered in *PEX2* or *GLIS3* (Table II), a rare nonsynonymous variant in *PEX2* (chr8:77900416:T/C, MAF = 0.16%, OR = 3.33, $P = 1.25 \times 10^{-2}$) and another in *GLIS3* (chr9:3898723:C/T, MAF = 0.14%, OR = 0.33, $P = 3.90 \times 10^{-2}$), were nominally associated with risk of ND (Supplementary Table VI). The remaining genes implicated in the present meta-analysis (*GABBR1*, *CHRNA2*, *ADAMTS1*, and *LINC00476*) were not supported by the rare variant analysis. Among genes previously implicated in the etiology of ND, a rare nonsynonymous variant in *DLC1* was nominally associated with ND (Supplementary Table VI), but the gene-based tests did not identify any significant aggregate associations with ND in previously identified susceptibility genes (*CHRNA3*, *CHRNA4*, *CHRNA5*, *CHRNA6*, *DLC1*, *DISC1*, *C14ORF28*, and *KLHL28*) (Table II).

Discussion

ND has a reported heritability of 40–70% [Kendler et al., 1999; Sullivan and Kendler 1999; Vink et al., 2005]. Although GWA studies have previously detected multiple loci associated with ND, even in aggregate they only account for a small fraction of the measured heritability of ND. In the present study, genome-wide meta-analyses were used to identify common SNPs associated with susceptibility for ND. Findings from these meta-analytic efforts were then further explored by using transcriptional expression data in human brains evaluate the possible regulatory effects of the implicated SNPs on gene expression. This study confirmed five previously reported loci (i.e., *CHRNA5-A3-B4*, *KLHL28*, *C14ORF28*, *DISC1*, and *DLC1*), identified a novel susceptibility gene *CACNA2D3* in the African data set as well as six other potential loci on chromosomes 1, 6, 8, and 9. We provided further evidence suggesting that these implicated SNPs altered mRNA expression levels of genes *CACNA2D3*, *HAX1*, *CHRNA2*, *ADAMTS1*, *GLS3*, *LINC00476*, and *PEX2* through eQTL effects in human brains. Analyses of rare variants conducted in the UCSF Family Alcoholism study provided further evidence for the implicating genes. Specifically, we searched for rare nonsynonymous variants occurring in the implicated genes in low-pass whole-genome sequencing data in the UCSF Family Alcoholism study, and identified four rare nonsynonymous variants in the *CACNA2D3*, *HAX1*, *PEX2* and *GLIS3* genes that conferred risk for ND, as well as an aggregate effect of rare nonsynonymous variants in

HAX1 associated with ND diagnostic status. Together, these results demonstrate the utility of meta-analysis, when supplemented with the analysis of expression-level data and sequence-based data, for accelerating the discovery of functionally relevant genes involved in the etiology of ND.

In the present study, a novel genome-wide significant association of rs56247223 on chromosome 3 with risk for ND was identified in the African samples. SNP rs56247223 is located in an intron of *CACNA2D3*, and was associated with mRNA expression levels of *CACNA2D3* in three human brain regions (cortex, frontal cortex, and putamen). In terms of its function, *CACNA2D3* encodes a protein member of the voltage-dependent calcium channel complex. Calcium channels mediate the influx of calcium ions into cells and have an important role in neurotransmission [Simms and Zamponi 2014]. Calcium channels have been widely implicated in the pathophysiology of substance dependence, and they are therapeutic targets for addiction medications [Addolorato et al., 2012; Zamponi 2016]. Several different types of calcium channels exist, and each typically consists of four subunits, of which the *CACNA2D3* gene encodes the alpha-2/delta subunit [Simms and Zamponi 2014]. Genomic aberrations of the genes encoding the alpha subunit of these channels have been associated with epilepsy and neuropsychiatric disorders such as autism and schizophrenia [Vergult et al., 2015]. This may be important given that smoking behavior is highly prevalent in schizophrenia patients [Dickerson et al., 2013]. Of direct relevance to the present study, the voltage-dependent calcium channel complex alpha-2 and delta subunit family member gene *CACNA2D1*, was previously associated with risk of ND [Gelernter et al., 2015]. With respect to the association of rs56247223 with ND in present study, the protective A allele of rs56247223 was associated with a decreased expression level of the gene *CACNA2D3* in human brain. This finding is consistent with the observation that expression of L-type high voltage-gated calcium channel alpha2/delta subunits increases after chronic nicotine administration in mouse brain [Hayashida et al., 2005]. *CACNA2D3* has also been implicated with other smoking phenotypes, including a robust association with smoking cessation, in a convergent analysis of three GWA data sets [Uhl et al., 2008]. Collectively, these results support *CACNA2D3* as a susceptibility gene and potential as a therapeutic target for the treatment of ND.

Despite this evidence, it should be noted that rs56247223 was not significantly associated with ND in the European ancestry cohort. Differences in genetic background may explain why an association was detected in the African but not in the European populations. Nonetheless, the detection of a rare nonsynonymous variant in the *CACNA2D3* gene confers a large risk (OR > 3) for ND in the UCSF Family Alcoholism study, of which the majority of subjects are of European ancestry, provides additional support that the gene plays a role in ND.

We found an association of the SNP rs1760803 with ND in the European ancestry cohorts. The mRNA expression levels of two nearby genes, *HAX1* and *CHRNA2*, in human brain tissue were found to be associated with this SNP. In the UCSF Family Alcoholism study, one rare nonsynonymous variant in *HAX1* was associated with an increased risk of ND, and the presence of rare variants in the *HAX1* gene was found to be associated with an increased risk of ND diagnostic status. The *HAX1* gene encodes a protein that often binds to several

proteins, including cortactin and the product of the polycystic kidney disease 2 gene. Mutations in the *HAX1* gene are responsible for autosomal recessive severe congenital neutropenia (SCN), and mutations in the HAX1 protein B isoform are associated with neuropathy in patients with SCN [Boztug et al., 2010]. The nicotine receptor subunit $\beta 2$ gene *CHRNA2*, which is also implicated in this region, has been shown to mediate an early physical and psychological response to nicotine [Ehringer et al., 2007]. Therefore more studies are warranted in order to determine which gene in this particular region is actually influencing the ND phenotype.

SNP rs17198023 was found to show consistent association with ND across all European ancestry cohorts. We further found that this SNP modulated mRNA expression level of the *ADAMTSL1* gene on chromosome 9 in human brain caudate basal ganglia. The ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type-1 repeats) like 1 gene (*ADAMTSL1*) encodes an ADAMTS family protein. In a previous GWA study, *ADAMTSL1* variants were shown to be associated with antidepressant drug response phenotype in depressed patients [Ising et al., 2009]. Additionally, systemic administration of FG7142, an anxiogenic drug and/or a pharmacological stressor that modulates GABA receptors, leads to an increase in mRNA for *ADAMTSL1* in the cortex and hippocampus of mice [Kurumaji et al., 2008; Kurumaji and Nishikawa 2012]. More studies will be needed, however exploring how this gene may potentially influence ND.

In the meta-analysis of European and African merged samples, there was a suggestive association found between ND and SNP rs12348139, an intron in gene *GLIS3*. This result was partially confirmed by the finding of a rare nonsynonymous variant in *GLIS3* in the UCSF Family Alcoholism study cohort that conferred a protective effect on ND. The *GLIS3* gene is highly expressed in brain [Cruchaga et al., 2013]. SNP rs12348139 was an eQTL site for the *GLIS3* gene in human brain. The *GLIS3* gene encodes a member of the GLI-similar zinc finger protein family, which is a nuclear protein with five C2H2-type zinc finger domains, and is recognized as a repressor and activator of transcription. The *GLIS3* gene has been identified via GWAS with two commonly used biomarkers, cerebrospinal fluid (CSF) tau and tau phosphorylated at threonine 181 (ptau), for Alzheimer's disease [Cruchaga et al., 2013], and also the risk of type 2 diabetes [Cho et al., 2012]. Notably, chronic nicotine administration exacerbates tau pathology in a transgenic model of Alzheimer's disease [Oddo et al., 2005], and cigarette smoking is correlated with a higher risk of Alzheimer's disease and type 2 diabetes [Cataldo et al., 2010; Spijkerman et al., 2014]. Thus, *GLIS3* may have a pleiotropic effect on these three disorders that could explain, in part the pathogenesis underlying the clinical link between cigarette smoking, and Alzheimer's disease, and type 2 diabetes. We also identified SNP rs10759883 in a long non-coding RNA *LINC00476* on chromosome 9 that was associated with ND. The minor risk allele C of rs10759883 was found to upregulate the expression level of *LINC00476* through eQTL effects in multiple human brain regions. Although the functional relevance of *LINC00476* is unclear, recent evidence suggests that non-coding RNAs play an important and dynamic role in transcriptional regulation, epigenetic signaling, stress responses, and plasticity in the nervous system [Sartor et al., 2012]. Therefore, the role of *LINC00476* in ND is worthy of further investigations.

Finally, two SNPs in the *GABBR1* gene were found to be suggestively associated with the risk of ND in the meta-analyses for both European and African populations. One of these SNPs, rs62392942, was also found to be associated with altered mRNA expression of *GABBR1* in human brain putamen. Further, predicted expression levels of the *GABBR1* gene in brain, estimated using the recently developed gene-based association method MetaXcan [Barbeira et al., 2016], were shown to be significantly associated with risk for ND. The *GABBR1* gene is functionally relevant to ND. More specifically, the gene encodes a receptor for gamma-aminobutyric acid (GABA), which is the main inhibitory neurotransmitter in the mammalian central nervous system [D'Souza and Markou 2013]. Evidence in animal studies suggests that both nicotine intake and nicotine seeking are attenuated when GABA neurotransmission is facilitated [D'Souza and Markou 2013]. Haplotypes of the *GABBR1* gene have also been identified with risk of ND in a previous study, and it has been further suggested that the *GABBR1* gene contributes to the risk of ND through interactions with *GABBR2* [Li et al., 2009]. The current study provides further genetic evidence suggesting a role for *GABBR1* variation in risk for ND.

ND is a complex and multi-faceted construct, and as a result it has been operationalized in a number of ways (e.g., DSM-IV diagnosis, Fagerström Test ND score). GWAS of these ND phenotypes have been pursued in multiple independent, large-scale cohorts, but few genomic regions have been consistently and mutually validated. The differences in phenotype definition and phenotypic heterogeneity could be partly responsible for that. In the present study, we used the DSM-IV diagnosis of ND, and thus, it is not surprising that we did not observe significant overlap in association signals previously reported in the GWAS of Fagerström Test ND score [Gelernter et al., 2015]. As noted, phenotypic heterogeneity could have also contributed to the lack of overlapping signals. The presence of co-morbid substance use disorders likely represents one such source of heterogeneity. For example, several of the contributing GWA cohorts were selected on other forms of substance use disorder (e.g., alcohol, cocaine dependence). It is likely that the etiologic factors, including genetic influences, that contribute to the development of ND in individuals with multiple substance use disorders differ, at least in part, from those factors that contribute to the development of ND in individuals with just ND. Unfortunately, data on co-occurring substance dependence diagnoses were not available across all study cohorts, and thus, could not be studied as covariates or as moderators in the association analysis. Nonetheless, the potential impact of polysubstance use on the observed associations, and results from GWAS of substance use disorders more broadly, is an important focus of study that we hope future studies will be able to address.

The findings of this study should be interpreted in light of several limitations. One limitation is that several findings did not exceed the traditional genome-wide significance threshold, however, follow-up analyses such as the eQTL analysis provided some additional support for these findings. The eQTL analyses did not employ genome-wide corrections for multiple testing because they were conducted to address the specific hypothesis that variants in a candidate gene are associated with expression levels when there is prior evidence of association. The second limitation is the lack of an “independent” validation sample. Recent large-scale genome-wide meta-analyses have implemented broadly a two-stage study design in which they perform the initial meta-analysis, pick a set of variants to carry forward, then

pursue replication attempts in sets of independent studies, and finally conduct a combined analysis of all available data. Because it has been argued that the validation effort in the meta-analysis of a two-stage design is not a true independent replication [Thomas et al., 2009] and considering the relatively small sample size in the present study, we pursued a single-stage rather than two-stage design with the aim at gathering exhaustively all available data to improve power for discovery..

In summary, we carried out a large-scale GWA meta-analysis on ND, and identified seven susceptibility signals for ND. Notably, our study provides several lines of evidence supporting key role for genes *CACNA2D3* and *GABBR1* in ND. The respective proteins of these genes are important for calcium channel and GABA neurotransmitter signaling, both of which are currently the main therapeutic targets for the treatment of ND. These findings not only elucidate the pathophysiology of ND, but also highlight potential therapeutic targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

B

AA_rs56247223

Figure 1. Association of SNP rs56247223 with ND and the eQTL effect on the *CACNA2D3* gene in human brain

(a) Forest plots of the meta-analysis results. (b) Regional association plots for rs56247223 in AFR samples. The relative location of annotated genes and the direction of transcription are shown in the lower portion of the figure. The chromosomal position is shown on the x-axis. The blue line shows the recombination rate (estimated from HapMap data of AFR sample) across the region (right y-axis), and the left y-axis shows the significance level of the SNP associations ($-\log_{10}P$). The color of the squares and circles indicate the LD status between the SNPs and the leading SNP. (c) The eQTL effect on the *CACNA2D3* gene in human brain cortex. The y-axis represents the normalized transcript expression level in the unit of RPKM. SNP: single nucleotide polymorphism; eQTL: expression quantitative trait locus; EUR: European; AFR: African; LD: linkage disequilibrium; RPKM: Reads Per Kilobase of transcript per Million mapped reads. SAGE: Study of Addiction: Genetics and Environment; ADGE: Alcohol Dependence GWAS in European- and African Americans; GASSC: The Genetic Architecture of Smoking and Smoking Cessation; ICGHD: International Consortium on the Genetics of Heroin Dependence; OZALC: Alcohol Research using Australian twins and their families. CIDR: Center for Inherited Disease Research), RATRP: Research on Alcohol and Tobacco Related Phenotypes) and ARC: Alcohol Related Conditions.

Table 1

Summary statistics for studies

study	dbGaP accession	array	EUR			AFR		
			case	control	No. of SNPs	λ_{gc}	case	control
SAGE	phs0000092.v1.p1	Illumina Human 1M	1048	1268	4524998	1.009	578	605
ADGE	phs000425.v1.p1	Illumina HumanOmni1-Quad	481	181	4261404	0.995	913	824
GASSC	phs000404.v1.p1	Illumina HumanOmni2.5	714	146	5174464	1.019	378	71
ICGHD CIDR	phs000277.v1.p1	Illumina Human660W-Quad	1318	1079	5298585	1.031	0	0
ICGHD RATRP	phs000277.v1.p1	Illumina Human610_Quad	101	661	4751941	1.017	0	0
ICGHD ARC	phs000277.v1.p1	Illumina HumanCNV370-Quad	306	77	4720266	1.004	0	0
OZALC	phs000181.v1.p1	Illumina HumanCNV370v1	3329	2165	6245839	1.039	0	0
UCSF	–	Whole-genome sequencing	918	921	8393347	1.005	0	0
Total			8215	6498	3,251,541	1.022	1869	1500
							5,227,780	1,003

dbGaP: the database of Genotypes and Phenotypes; EUR: European population; AFR: African population; No. of SNPs: number of single nucleotide polymorphisms; SAGE: Study of Addiction: Genetics and Environment; ADGE: Alcohol Dependence GWAS in European- and African Americans; GASSC: The Genetic Architecture of Smoking and Smoking Cessation; ICGHD: International Consortium on the Genetics of Heroin Dependence; OZALC: Alcohol Research using Australian twins and their families; CIDR: Center for Inherited Disease Research), RATRP: Research on Alcohol and Tobacco Related Phenotypes) and ARC: Alcohol Related Conditions.

Table II

Summary statistics for the gene-based test of rare variants in the UCSF Family Alcoholism study

CHR	BEGIN	END	GENE	Variant No.	P
1	231762651	232172555	DISC1	16	1.72E-01
1	154245225	154247908	HAX1	3	2.80E-02
1	154542792	154548331	CHRNA2	6	1.00E+00
3	54156747	55107846	CACNA2D3	14	7.71E-01
6	29571342	29600126	GABBR1	2	1.90E-01
8	12943795	13357567	DLC1	29	7.29E-01
8	77895667	77913120	PEX2	9	3.35E-01
9	18533279	18906866	ADAMTSL1	20	1.00E+00
9	3828355	4286526	GLIS3	20	3.00E-01
14	45369682	45374747	C14ORF28	3	5.13E-01
14	45403373	45414662	KLHL28	6	1.00E+00
15	78873272	78885473	CHRNA5	3	5.33E-01
15	78885473	78911261	CHRNA3	5	5.66E-01
15	78917316	79012516	CHRNA4	10	3.96E-01
20	61978149	62005888	CHRNA4	12	6.71E-01

CHR: chromosome; BEGIN: the start position of gene; END: the end position of gene; Variant No.: the valid available rare nonsynonymous variants. P: the EMMAX SKAT association P-value.