Addiction

RESEARCH REPORT

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Gamma-aminobutyric acid receptor genes and nicotine dependence: evidence for association from a case–control study

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ABSTRACT

Aims The gamma-aminobutyric acid receptor A (*GABRA*) gene clusters on chromosomes 4 and 5 have been examined previously for their association with alcohol and drug dependence phenotypes. Compelling evidence suggests that *GABRA2* is associated with alcohol and drug dependence. However, no study has investigated whether genes in the $GABA_A$ gene clusters are associated with nicotine dependence, an important phenotype with a high correlation to persistent smoking, the single most preventable cause of mortality world-wide. **Design** Using data on 1050 nicotine-dependent cases and 879 non-dependent smoking controls, we used logistic regression to examine the association between single nucleotide polymorphisms (SNPs) in 13 genes in the $GABA_A$ receptor system as well as *GABBR2* (a $GABA_B$ gene). **Findings** We found evidence for association between four SNPs in *GABRA4*, two SNPs in *GABRA2* and one SNP in *GABRE* with nicotine dependence. These included a synonymous polymorphism in *GABRA2* (rs279858), lying in a highly conserved region, which has been shown previously to be associated with alcohol and drug dependence. A non-synonymous polymorphism (rs16859834/rs2229940) in *GABRA4*, also highly conserved, was associated at *P*-value of 0.03. Significant haplotypes associated with nicotine dependence were found for *GABRA2*. No evidence for epistatic interactions were noted. Our study did not find evidence for an association between *GABBR2* gene and nicotine dependence. **Conclusions** Given the potential role of compounds that enhance GABAergic neurotransmission in smoking cessation research, these findings have enormous potential for informing the wider field of addiction research.

Keywords Association, GABA, nicotine dependence, NICSNP.

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INTRODUCTION

An estimated 44.5 million adults in the United States report having smoked cigarettes [1]. Approximately one of every five preventable deaths in the United States is a consequence of tobacco smoking [2]. Tobacco use also contributes to 1.5 million cancer-related deaths per year across the world, equivalent to 20% of all cancer-related deaths in 2005 [3]. Currently, 1 billion men (35% in developed countries and 50% in developing countries) and 250 million women (22% in developed and 9% in developing countries) smoke cigarettes [4]. Given these alarming statistics, there is a persisting public health demand for studies that examine the biological and environmental underpinnings to persistent smoking and nicotine dependence.

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Numerous studies of adult twins [5–7] have revealed that diverse stages of tobacco smoking, from experimentation to regular use and nicotine dependence, are moderately to strongly heritable: smoking initiation (heritability $h^2 = 40–80\%$) [6,8–15], regular tobacco smoking ($h^2 = 63\%$) [[16,17], smoking persistence ($h^2 = 60–80\%$) [18–20] and nicotine dependence ($h^2 = 60\%$) [8,21–23].

GABA (gamma-aminobutyric acid) is the major inhibitory neurotransmitter system. The genes encoding the $GABA_A$ (gamma-aminobutyric acid receptor A) and $GABA_B$ (gamma-aminobutyric acid receptor B) receptors are likely to be involved in aspects of nicotine dependence. For instance, dopamine release in the nucleus accumbens may be regulated by GABAergic transmissions [24,25]. In particular, the $GABA_B$ agonist Baclofen has been shown consistently to reduce the rewarding effects of nicotine in animal models, suggesting its potential as a treatment for nicotine dependence [24,26,27]. In one human laboratory study, individuals who were not trying to quit smoking cigarettes, when administered Baclofen, reported more negative subjective reactions (e.g. harshness) during smoking and also attributed sedative-like effects to the medication [28].

The $GABA_A$ genes have long been studied for their association with psychiatric phenotypes, especially alcohol and drug dependence (*GABRA2*) [29], schizophrenia (chromosome 5 cluster) [30–32] and autism (*GABRA4* and *GABRB1*) [33,34]. With data from the Collaborative Study on the Genetics of Alcoholism (COGA), Edenberg and colleagues have reported previously an association between alcohol dependence and multiple single nucleotide polymorphisms (SNPs) in *GABRA2* [35]. This finding was replicated subsequently across independent US [36], Russian [37] and German [38] data sets. Extensions of this work have also demonstrated the high probability that *GABRA2* confers a general vulnerability to comorbid alcohol and drug dependence [39,40] and also to a more general spectrum of externalizing disorders [41]. With the exception of a recently reported association between *GABARAP* ($GABA_A$ receptor-associated protein) and nicotine dependence in Caucasians [42], surprisingly little is known of the relationship between $GABA_A$ genes and nicotine dependence.

In the same study, a gene in the receptor B cluster (*GABBR2*) was also found to be associated with nicotine dependence in European and African American populations [43]. Due to its proposed influence on the reward threshold of nicotine, this receptor class is being investigated actively by tobacco cessation researchers.

Given the global burden of tobacco smoking and the biological importance of $GABA_A$ genes in the etiology of multiple substance use disorders, we investigated the association between 154 SNPs in $GABA_A$ genes and nicotine dependence as assessed by the Fagerström Test for Nicotine Dependence (FTND) [44,45] using data from the NICSNP (Nicotine Single Nucleotide Polymorphism Study) project, a collaborative genetic study that included a genome-wide and candidate gene association component. We also attempted to replicate the previously observed association between 28 SNPs in the *GABBR2* gene and FTND in our study.

MATERIALS AND METHODS

Participants

Study participants for NICSNP were selected from two independent and ongoing community-based samples ascertained in the United States and Australia. Participants from the United States were recruited as part of the Collaborative Genetic Study of Nicotine Dependence (COGEND) from sites in St Louis, Detroit and Minneapolis [46]. The Australian participants were drawn from the Nicotine Addiction Genetics (NAG) [47,48] study, and included families identified using two cohorts of the Australian Twin Panel, which included spouses of the older twins. From both studies, only participants who had reported a history of smoking (100 or more cigarettes in their life-time), when queried by telephone interview, were eligible for inclusion in the NICSNP study. Life-time smokers from COGEND and NAG, respectively, with an FTND score of '0' for a self-reported period of smoking were eligible as controls. Participants reporting themselves to be current smokers with an FTND score of 4 or greater currently (COGEND) or meeting this criterion for a self-reported period of heaviest smoking (NAG) were eligible as cases. Cases and controls were unrelated. Only Caucasian participants were included from the US sample, as ethnic minorities of the United States do not apply readily to Australian samples. The minorities of the latter consist of Indigenous Aboriginal populations that are not accessible to investigators without specialized permission. Thus, for consistency, individuals of European ancestry were selected from both samples. The Institutional Review Boards approved the protocol for both studies. Blood samples collected for DNA extraction were submitted along with electronic phenotypic and genetic data for both studies to the National Institute on Drug Abuse (NIDA) Center for Genetic Studies, which manages the sharing of research data according to guidelines of the National Institutes of Health.

Measures

Participants of European–Caucasian ancestry with a life-time history of smoking 100 or more cigarettes and scoring 4 or more on the FTND (with scores ranging from 0 to 10) were designated as nicotine-dependent cases. Previous studies have shown the FTND to be associated

with biological indices of nicotine dependence [44]. Sensitivity–specificity (ROC) analyses by Breslau & Johnson [49] supports this cut-off as an index of nicotine dependence. In addition, from early work using data obtained from a survey of adult Australian twins, we observed that monozygotic (MZ) twin smokers reporting a history of FTND = 0 were less likely than never smokers to have a nicotine-dependent co-twin, supporting our control definition. The NICSNP sample consisted of 1929 participants, 1050 designated as cases and 879 as controls. Approximately 24% and 76% of the cases (and 8% and 92% of the controls) were from Australia and the United States, respectively. Overall, the sample included more women (62%) than men. Cases had a mean age of 37.7 years (range 25–82) and were 44% male, while controls were, on average, aged 36.7 years (range 25–82) and were 30% male.

Genotyping

The procedure for candidate gene selection has been detailed elsewhere [46,50]. Briefly, genes were selected by a panel of expert members of the NIDA Genetics Consortium for their putative biological significance in addiction. Candidate genes (448 in all) were divided into 'A' and 'B' lists based on their biological relevance and prior published evidence for association. For example, the A list included the *GABBR2* gene, while the B list included the $GABA_A$ receptor genes.

For this paper we utilize data on 154 SNPs in $GABA_A$ [listed by chromosomal location, name and (number of SNPs genotyped)] as follows: on chromosome 1: *GABRD* (2); on chromosome 4: *GABRA2* (6), *GABRA4* (27), *GABRB1* (17); on chromosome 5: *GABRA1* (11), *GABRA6* (14), *GABRB2* (14), *GABRG2* (13); on chromosome 6: *GABRR1* (20); on chromosome 15: *GABRA5* (6), *GABRB3* (14); and on chromosome X: *GABRE* (7), *GABRA3* (3). We also analyzed 28 SNPs in *GABBR2* on chromosome 9.

For individual genotyping, we designed custom high-density oligonucleotide arrays to interrogate SNPs selected from candidate genes, as well as quality control SNPs. Each SNP was interrogated by 24 25-mer oligonucleotide probes synthesized on a glass substrate. The 24 features comprise four sets of six features interrogating the neighborhoods of SNP reference and alternate alleles on forward and reference strands. Each allele and strand is represented by five offsets: −2, −1, 0, 1 and 2, indicating the position of the SNP within the 25-mer, with zero being at the 13th base. At offset 0 a quartet was tiled, which includes the perfect match to reference and alternate SNP alleles and the two remaining nucleotides as mismatch probes. When possible, the mismatch features were selected as purine nucleotide substitution for a purine perfect matched nucleotide and a pyrimidine nucleotide substitution for a pyrimidine perfect matched nucleotide. Thus, each strand and allele tiling consisted of six features comprising five perfect matched probes and one mismatch.

Details regarding SNP selection and genotype cleaning are presented in a related paper [50]. All exonic SNPs (regardless of frequency) and SNPs within 2 kb of promoters (minor allele frequency greater than 4% in European Americans) were selected. Tag SNPs from pre-defined linkage disequilibrium (LD) bins were then selected to cover the gene. A quality metric (discordant calls between the Perlegen platform and non-Perlegen HapMap genotypes) was used for genotype cleaning. A series of bootstrapped regression trees, with cross-validation, were used to determine genotype quality. Additionally, the software package STRUCTURE was used to examine evidence for population admixture using 289 high-performance SNPs across 1050 cases and 879 controls; no support in favor of population admixture between cases and controls was found.

Genotypes were coded as 0, 1 or 2, indicating the number of copies of the minor allele. Cluster plots were examined to ensure minimal errors in genotype calls. SNPs with call rates less than 90% were excluded from analyses, as were SNPs with Hardy–Weinberg *P*-values less than 0.05. In general, genotyping quality was good and call-rates were close to 98–99% for all SNPs in these genes. It is worth noting, however, that rs279858 in *GABRA2* had some ambiguous calls between heterozygous and homozygous genotypes, and had a call rate of 94% which, while acceptable, was somewhat lower. We anticipate re-coding genotypes for this polymorphism in the future. Hardy–Weinberg equilibrium *P*-values were greater than 0.05 for all SNPs.

Data analysis

Logistic regressions

Logistic regressions, using SAS version 8 [51], were used to examine the association between individual SNPs and case–control status (dependent variable). Controls for gender (0 = male, 1 = female) and site (1 = Australia, 0 = United States) were also included. As noted in a previous publication [48], after the addition of site as a control measure, age did not contribute significantly as a covariate and was consequently dropped from analyses. The primary statistic was a 2-degree of freedom test of the main effect of genotype and the interaction between genotype and gender.

Haplotype trend regression

Haplotype association is often used to explore further the association between a phenotype and multiple SNPs

across a gene by using conformations of SNPs that segregate together. We performed haplotype association analysis using sliding windows of two, three and four SNPs only when single SNPs in a candidate gene had *P*-values of 0.01 or less. SNPs were selected if they were not in LD (r^2 of 0.90 or less) with any other SNP in the gene. Where possible, SNPs with the largest minor allele frequency or with greater evidence for association (i.e. more significant *P*-values) were selected to represent the LD bin. The haplotype trend regression method [52] was implemented in SAS (PROC HAPLOTYPE) and modified to include covariates (gender and site). Briefly, PROC HAPLOTYPE in SAS Genetics [53] was used to compute two, three and four SNP haplotype configurations and their frequencies, which were then entered into a stepwise logistic regression model, predicting case status, along with covariates of gender and site. Haplotypes significant at *P*-value of 0.05 or less were retained and their joint test Wald χ^2 *P*-value was used to test for statistical significance.

Gene–gene effects

We used logistic regression analyses to investigate additive and interactive effects between SNPs identified in our best-fitting multi-locus model. SNPs selected for haplotyping (i.e. thinned by LD) were incorporated into a model along with sex and site as fixed covariates. A stepwise procedure was used to retain SNPs significant at alpha = 0.05. Next, interactions between SNPs retained by the stepwise model were incorporated into the model, including three-way interactions with sex.

Multiple testing

To account for the effects of multiple testing, *Q*-values for each SNP were calculated using QVALUE in the software package R (available at http://www.faculty.washington.edu/~jstorey/qvalue/) [54]. The false discovery rate for our analyses was set at 0.05 [55], accounting for multiple-testing across 182 SNPs. Unlike the previous report by Saccone *et al.* [48], no weights were ascribed to specific genes in this report, as the a priori hypothesis of the current study was to analyze only SNPs genotyped in the $GABA_A$ and $GABA_B$ systems.

RESULTS

Logistic regression analyses

After controlling for gender and site, both of which were associated significantly with case status (i.e. a positive association between case status and male gender and a negative association between case status and site), six SNPs in the $GABA_A$ gene cluster on chromosome 4 were associated significantly with an FTND score of 4 or more (case) when compared with individuals who had smoked 100 or more cigarettes in their life-time but scored 0 on the FTND. Four of these SNPs (rs11731576, rs2280072, rs3762607, rs3762611) were in *GABRA4* (Table 1), while two SNPs (rs279858, rs573400) were in *GABRA2* (Table 2), which lies about 530 kb upstream from *GABRA4* and *GABRB1*. For *GABRA4*, rs11731576 and rs2280072 were in high LD (Fig. 1), as were rs3762607 and rs3762611. In addition to these polymorphisms, rs16859834 (mapped recently to rs2229940), a non-synonymous polymorphism affecting a Met26Leu (methionine to leucine at amino acid position 26) change was associated with FTND at a *P*-value < 0.05. This SNP was, however, not in high LD (r^2 = 0.04–0.72) with neighboring SNPs, all of which had significant results. The two significant SNPs in *GABRA2* were in moderate LD with each other and with rs6833452 (Fig. 1), which was significant at a trend level. In addition, one SNP in *GABRE* on Xq28 was also associated at *P*-value of 0.006. Furthermore, these SNPs, with the exception of rs573400, had *Q*-values less than 0.20.

Suggestive association at *P*-values nearing 0.05 (see Supplementary material Table S1) was also seen for a number of SNPs in *GABRR1* as well as for two SNPs in *GABRG2*. Previous work by Beuten *et al.* [43] has shown there to be an association between FTND and the *GABBR2* (gamma-aminobutyric acid receptor B, subunit 2) gene on chromosome 9. In our study, we tested for this relationship using 28 SNPs and did not find any evidence for association between SNPs in *GABBR2* and nicotine dependence.

Haplotype trend regressions

SNPs in *GABRA4*, *GABRA2* and a SNP in *GABRE* were associated with nicotine dependence at alpha = 0.01 and were pursued in haplotype association analyses. After accounting for high LD in SNPs typed in *GABRA4* (see Fig. 1), *GABRA2* (see Fig. 1) and *GABRE* (see Fig. 1), we performed haplotype trend regression (HTR) analyses on 11, four and four SNPs in *GABRA4*, *GABRA2* and *GABRE*, respectively (denoted by an asterisk in Tables 1–3). For *GABRA4*, the global *P*-values for a two-SNP, three-SNP and four-SNP model, all of which included our most significant single SNP rs3762611, were 0.0006, 0.001 and 0.0029, respectively. As the *P*-value for the single SNP association analysis was 0.0009, haplotypes were not informative for *GABRA4*, as they were driven by the effects of this single SNP and did not extend across the gene.

Similarly, haplotype configurations with rs1061420, rs1061418, rs2256882 and rs1158605 in *GABRE* yielded a global χ^2 of 4.67 (*P*-value of 0.031 for 1 degree of freedom), which was comparable to the single-SNP association *P*-value of 0.0062 for rs1061418.

Table 1 Single nucleotide polymorphism (SNP) association results for gamma-aminobutyric acid receptor 4 (GABRA4) on chromosome 4p12–13.

Marker	*Position (bp)*	*Minor allele*	*Minor allele frequency (case)*	*Minor allele frequency (control)*	*Primary P-value*	*Genotype P-value*	*Genotype × sex P-value*	*Q-value(FDR)*
rs13135800	46736264	G	0.29	0.28	0.694	0.7338	0.436	0.99
rs7683552*	46739193	C	0.23	0.23	0.994	0.9456	0.937	0.99
rs17598928	46751328	T	0.29	0.29	0.695	0.7112	0.445	0.99
rs7691100	46762882	T	0.29	0.29	0.709	0.7262	0.456	0.99
rs10033500*	46763616	T	0.28	0.28	0.601	0.8215	0.327	0.99
rs9291296	46763878	G	0.29	0.28	0.631	0.6071	0.423	0.99
rs9291297	46764036	C	0.28	0.26	0.638	0.5535	0.465	0.99
rs956378*	46766674	T	0.11	0.10	0.866	0.6002	0.883	0.99
rs953380	46766741	G	0.28	0.27	0.642	0.7325	0.384	0.99
rs12506608	46767533	C	0.23	0.23	0.997	0.9565	1	0.99
rs1512139	46767705	T	0.47	0.48	0.733	0.7975	0.457	0.99
rs9291298*	46767765	G	0.47	0.49	0.673	0.8771	0.381	0.99
rs3920214	46767831	T	0.11	0.10	0.877	0.6696	0.768	0.99
rs17599074	46768052	C	0.29	0.28	0.648	0.6861	0.405	0.99
rs10004905	46768814	C	0.47	0.48	0.754	0.8668	0.464	0.99
rs4637372	46768957	A	0.47	0.48	0.714	0.8496	0.425	0.99
rs17599102	46769762	T	0.29	0.27	0.749	0.6124	0.578	0.99
rs16859700*	46770032	C	0.03	0.02	0.393	0.6192	0.2	0.99
rs7660336	46770596	G	0.47	0.48	0.680	0.8257	0.396	0.99
rs17599186*	46781946	A	0.24	0.24	0.980	0.8491	1	0.99
rs1160093*	46790698	C	0.42	0.41	0.876	0.9789	0.608	0.99
rs17599367*	46811644	A	0.12	0.11	0.747	0.5758	0.598	0.99
rs11731576*	46834584	T	0.08	0.11	**0.00251**	0.001	0.0438	0.12
rs2280072	46836098	C	0.08	0.11	**0.00265**	0.001	0.0401	0.12
†rs16859834*	46836294	T	0.39	0.36	**0.0327**	0.0727	0.0593	0.51
rs3762607	46837266	G	0.07	0.09	**0.00122**	0.0006	0.0343	0.12
rs3762611*	46838216	A	0.07	0.09	**0.000922**	0.001	0.075	0.12

Bold type: statistically significant at $P < 0.05$. FDR = false discovery rate; bp: base pairs. *SNP selected for haplotype trend regression. †Recently mapped to rs2229940, a non-synonymous polymorphism.

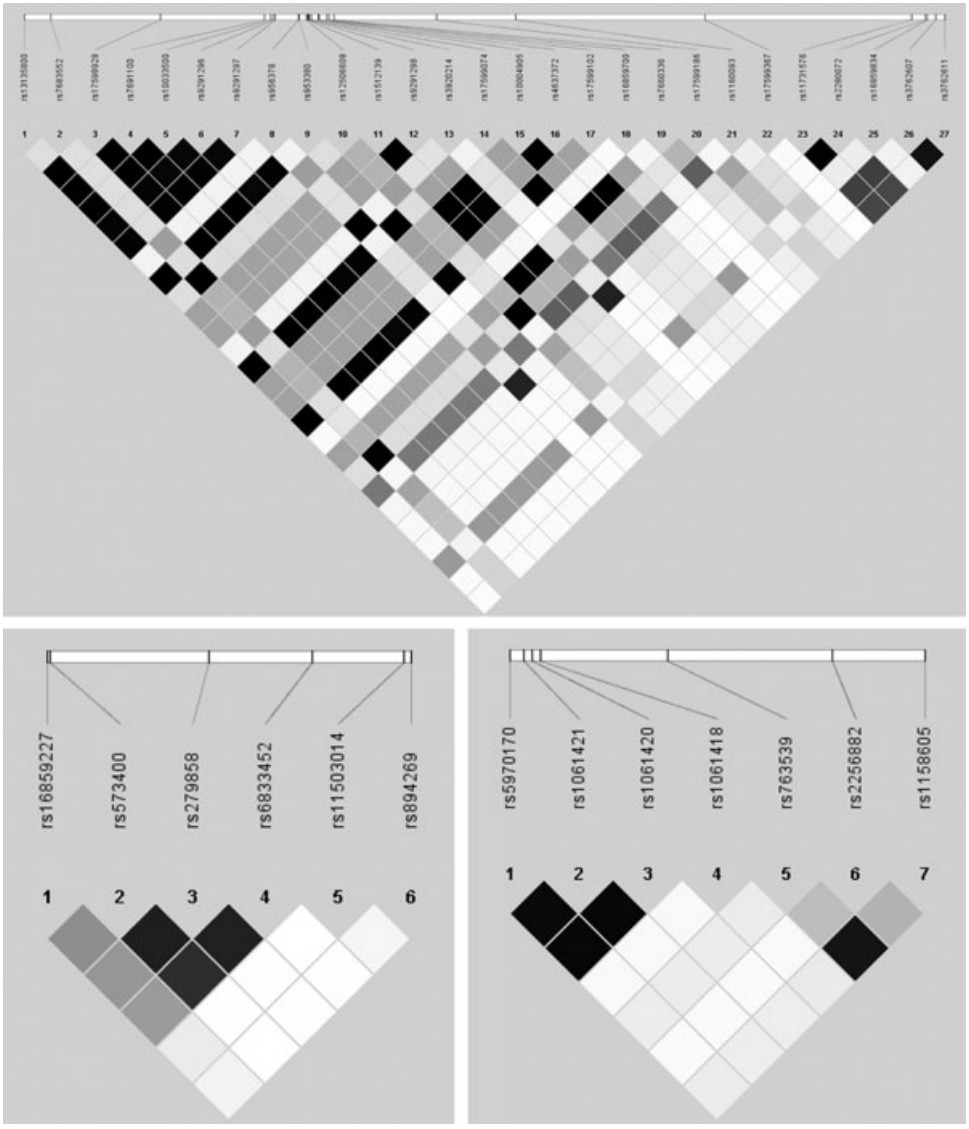

Figure 1 Linkage disequilibrium plots showing graphical extent of R^2 across single nucleotide polymorphisms (SNPs) in gamma-aminobutyric acid receptor 4 (GABRA4) (top panel), GABRA2 (bottom left of page) and GABRE (bottom right of page). White to black represents increasing linkage disequilibrium (LD) in cases and controls combined (estimates available upon request), where the black squares represent $R^2 \approx 1.0$

In contrast, we found informative haplotypes for *GABRA2* (Table 4). A three-SNP haplotype that included rs279858, the synonymous polymorphism associated most significantly with nicotine dependence, was associated at global *P*-value of 0.0003, which was a magnitude greater than the single SNP effect. Individuals with this haplotype configuration (C–C–T at rs279858–rs11503014–rs894269) were 3.2 [95% confidence interval (CI) 1.71–5.95] times more likely to have FTND scores ≥ 4 relative to the controls with FTND = 0.

Gene–gene effects

All SNPs short-listed for haplotype association analyses were entered into a multivariate logistic regression model, with gender and site. Only rs3762611 and rs279858, the strongest signals for *GABRA4* and *GABRA2*, respectively, were retained. rs1061418 in *GABRE* did not contribute additionally to predicting case–control status when SNPs in *GABRA4* and *GABRA2* were in the model. Next, the main effects of these SNPs, their interactions with each other, with gender and a three-way interaction (rs3762611*rs279858*gender) were introduced into the model. Stepwise regression retained only the main effects of each SNP and an interaction between rs279858 and gender [odds ratio (OR) 2.09, with ORs for rs3762611*rs279858*gender = 1.03, for gender*rs3762611 = 0.57 and for rs3762611*rs279858 = 0.67]. We also re-ran the full multivariate model separately in men and women. In

Table 2 Single nucleotide polymorphism (SNP) association analyses for gamma-aminobutyric acid receptor 2 (GABRA2) on 4p12–13.

Marker	*Position (bp)*	*Minor allele*	*Minor allele frequency (case)*	*Minor allele frequency (control)*	*Primary P-value*	*Genotype P-value*	*Genotype × Sex P-value*	*Q-value (FDR)*
rs16859227*	46091533	T	0.26	0.25	0.572	0.3189	0.755	0.99
rs573400	46092994	C	0.45	0.42	**0.00887**	0.0024	0.0132	0.24
rs279858*	46155521	C	0.45	0.41	**0.00471**	0.0014	0.0188	0.18
rs6833452	46195659	C	0.45	0.42	0.0393	0.0890	0.0607	0.51
rs11503014*	46231793	G	0.30	0.30	0.896	0.9838	0.639	0.99
rs894269*	46234540	T	0.17	0.15	0.343	0.5138	0.192	0.94

Bold type: statistically significant at $P < 0.05$. FDR = false discovery rate; bp: base pairs. *SNP selected for haplotype trend regression.

Table 3 Single nucleotide polymorphism (SNP) association analyses for gamma-aminobutyric acid receptor (GABRE) on Xq28.

Marker	*Position (bp)*	*Minor allele*	*Minor allele frequency (case)*	*Minor allele frequency (control)*	*Primary P-value*	*Genotype P-value*	*Genotype × sex P-value*	*Q-value (FDR)*
rs5970170	150792816	C	0.12	0.12	0.302	1	0.122	0.99
rs1061421	150793164	A	0.12	0.12	0.2	0.703	0.0794	0.83
rs1061420*	150793340	G	0.12	0.12	0.078	0.763	0.0252	0.64
rs1061418*	150793548	A	0.13	0.12	**0.00615**	0.0325	0.0179	0.19
rs763539	150796539	G	0.33	0.36	0.189	0.273	0.144	0.80
rs2256882*	150800390	A	0.13	0.14	0.722	0.429	0.868	0.99
rs1158605*	150802542	G	0.34	0.37	0.172	0.336	0.107	0.77

Bold type: statistically significant at $P < 0.05$. FDR = false discovery rate; bp: base pairs. *SNP selected for haplotype trend regression.

Table 4 Haplotype trend regression analyses for gamma-aminobutyric acid receptor 2 (GABRA2) (rs16859227–rs279858–rs11503014–rs894269).

Markers	*Haplotype*	*Case frequency*	*Control frequency*	*Global P-value*	*Odds ratio*
rs16859227–rs279858	C–T	0.54	0.58	0.0204	0.78 (0.56–0.95)
rs11503014–rs894269	C–T	0.15	0.13	0.0403	1.55 (1.04–2.32)
	G–T	0.01	0.02	0.0403	0.12 (0.03–0.49)
rs279858–rs11503014–rs894269	C–C–T	0.08	0.06	**0.000259**	3.19 (1.71–5.95)
rs16859227–rs279858–rs11503014–rs894269	C–C–C–T	0.07	0.05	0.00662	2.34 (1.27–4.32)

Haplotype associated most significantly with case–control status is shown in bold type.

women, after controlling for site, none of the SNPs were retained in the multivariate model (OR for SNP main effects ranged from 0.56 to 1.58). In men, however, in addition to rs3762611 (OR 2.15) and rs279858 (OR 1.51), rs16859834 (or rs2229940, OR 1.34), a non-synonymous polymorphism in *GABRA4*, and rs1061418 (OR 1.42) in *GABRE* were also retained. However, no two-, three- or four-way interaction was significant. Therefore, our analyses suggest that SNPs in *GABRA4*, *GABRA2* and *GABRE* have significant main effects on case status, primarily in men, with no evidence for epistatic interactions.

DISCUSSION

Based on evidence from the literature on substance abuse/dependence, we sought to examine the association between genes in the $GABA_A$ clusters on chromosomes 4, 5 and 15 as well as genes on chromosomes 1, 6 and X and one gene, *GABBR2*, on chromosome 9, and nicotine dependence. Keeping the caveat of multiple testing in mind, ours is the first study to reveal an association between *GABRA4* and risk for nicotine dependence, as assessed by an FTND score ≥ 4. A non-synonymous SNP (rs16859834/rs2229940) in *GABRA4* was associated with nicotine dependence. Furthermore, a synonymous polymorphism in *GABRA2*, which was implicated previously in associations with general vulnerability to substance use disorders, was found to be correlated with risk for nicotine dependence.

Our findings may be viewed with the following caveats in mind. Our control sample consisted of people who were exposed to smoking (smoked at least 100 cigarettes) but who developed none of the indicators of dependence (FTND = 0). We did not include individuals who were regular smokers and reported FTND scores of 1–3. Additionally, our sample consisted of individuals of European ancestry from the United States and Australia, and may not be extrapolated to other populations. Also, fewer cases and controls were ascertained from Australia than the United States. However, results were largely unchanged when conducting analyses in the US sample only. Another limitation of our study is that some candidate genes received inadequate SNP coverage or were not genotyped (e.g. GABRG1)—we hope to genotype these genes in the future.

Ours is the first study to report an association between multiple genes in the $GABA_A$ receptor genes and nicotine dependence. In a previous report, Saccone and colleagues [48] identified SNPs in *GABRA4* in this data set, from a total of 3713 SNPs in 348 genes, for their association with nicotine dependence. rs3762611 and rs3762607 (in *GABRA4*), our most significant SNPs, were ranked 6th and 10th, respectively, while rs279858 (in *GABRA2*) was ranked 27th. However, this is the first paper to perform comprehensive analyses (including haplotype association and interaction tests) focused upon the entire receptor system.

The primary *P*-values should be interpreted with caution, as we tested 182 SNPs. A Bonferroni correction (alpha/182) would be too conservative, as many of the SNPs are in LD and, moreover, is intended to guarantee that the probability that there are one or more false positives is less than alpha. We have six SNPs with a *Q*-value less than 0.2. We would expect 80% of them to be true positives. Accordingly, there is strong evidence that $GABA_A$ receptor genes are involved in nicotine dependence, although replication studies are needed to understand their exact role. Additionally, as noted recently by Curtis and colleagues, SNPs implicated previously in independent samples (e.g. rs279858) should be afforded additional attention compared with SNPs selected in genome-wide association studies or other genes in receptor families [56].

The $GABA_A$ genes encode the multimeric transmembrane chloride-gated ion channels that are receptors for the primary class of inhibitory neurotransmitters (gamma-aminobutyric acid) in the brain [57]. The most commonly occurring receptor in the human brain consists of 2 A, 2 B and 1 G subunits. In the human genome $GABA_A$ genes cluster on four chromosomes, 4, 5, 15 and X, with remarkable consistency in the organization of the individual subunit-encoding genes in each cluster. It has been posited that these clusters may have arisen as the consequence of a duplication of an ancestral cluster during chordate evolution [58–62].

We are not aware of any studies in humans that have identified an association between *GABRA4* and substance-dependence vulnerability. A majority of the research on *GABRA4* has focused on autism, where researchers have demonstrated an association between *GABRA4* independently, and interactively with *GABRB1*, on risk for autism [33,34]. It is noteworthy that rs16859834 (rs2229940), the non-synonymous SNP identified in our study for its association with nicotine dependence, has been implicated previously by these investigators for its association with autism. The SNP also resides in a region of fairly high evolutionary conservation and therefore may be of functional significance. However, in our study the association between *GABRA4* and nicotine dependence was due largely to a series of SNPs in high LD with each other. Haplotype association analyses did not reveal this association to extend across the gene even after accounting for LD.

In contrast to *GABRA4*, there is an abundance of evidence supporting the role of *GABRA2* in substance dependence vulnerability. Edenberg *et al.* [35] have reported an association between this gene and alcoholism, as have

others [36–38]. Recent studies have found this association to extend to alcoholism and comorbid illicit drug dependence [39,40]. In a majority of these studies, rs279858 was genotyped and demonstrated to have one of the most significant *P*-values. This synonymous polymorphism lies in exon 5 of *GABRA2*, in a region of very high evolutionary conservation, therefore alluding to its biological importance. Despite being 'silent', a synonymous polymorphism could have important effects on gene transcription via the modification of splicing sites. Work on the importance of silent polymorphisms in other genes has shown important transcriptional and translational modifications due to rare codon substitutions, even if they do not produce a change in valence [63]. An equally likely hypothesis is that rs279858 is in LD with a functional polymorphism, although no functional SNPs have been identified in *GABRA2* to date.

Important gender differences were noted for rs279858 (1.47 versus 1.03), rs573400 (1.42 versus 0.99) and for rs3762607 (2.16 versus 1.21). For each of these SNPs, the association between the risk allele and case status was significant in men, but did not meet statistical significance in women. To what extent this reflects a true gender-specific effect versus an overrepresentation of male cases can be disentangled only through careful replication studies in other samples. In analyses performed separately in men and women, none of the SNPs were particularly informative indices of risk for nicotine dependence. It is also plausible that environmental contributors to variance in smoking may play a more critical part in women than in men—twin studies have suggested the role of shared environmental factors on cigarette smoking, particularly in women [6,18], although whether this gender difference impacts individual differences in the FTND is not well established.

While we did not find any evidence for epistatic interactions between *GABRA4* and *GABRA2*, we did find strong additive influences from both genes. Logistic regression analyses demonstrated that both *GABRA4* and *GABRA2* had significant independent effects, both being more significant in men than in women. With the exception of the research on autism, few studies of the $GABA_A$ receptor genes have identified epistatic interactions for this gene family. This, however, does not rule out the possibility that the SNPs identified in *GABRA2* and *GABRA4* interact with SNPs in other receptor systems. We are particularly interested in interactions between GABAergic and glutamatergic SNPs, as the combined effects of these neurotransmitter systems on the reinforcing effects of nicotine is being investigated actively in animal models [24].

It is also noteworthy that we were unable to replicate the association between *GABBR2* and nicotine dependence, a finding reported previously by Beuten and colleagues [43] using pedigree-based association analyses. The gene was characterized adequately by us; however, the possibility still exists that differences in phenotypic definitions may have contributed to our negative findings. While Beuten and colleagues used a continuous FTND score in families ascertained for smoking, we used a case–control paradigm where regular smokers with an FTND of 0 were designated as controls. Additionally, we did not type any SNPs in *GABARAP*, for which an association with nicotine dependence has also been reported by this group.

The identification of genes in the GABA system is especially relevant to future efforts targeted at smoking cessation. Work by Markou and colleagues in rodent models demonstrates the effect of the GABA transaminase inhibitor GVG and GABA agonists such as Baclofen and CGP44532 on reducing nicotine consumption under a fixed-ratio reinforcement schedule [24,26,27]. However, these most promising GABA-related pharmacotherapies for nicotine dependence stem from $GABA_B$-agonists, such as Baclofen. The role of $GABA_A$ genes, however, are of possible etiological importance to a general vulnerability to substance dependence. Animal models have identified the receptor encoded by the *GABRA2* gene to be an important mediator of the anxiolytic properties of benzodiazepines [64,65]. While current medications for alcohol withdrawal have complex and indirect effects on $GABA_A$ receptors, the role of $GABA_A$-genes on smoking cessation and treatment of other substance use disorders remains to be validated [66].

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Supplementary material

The following supplementary material is available for this article online:

This material is available as part of the online article from http://www.blackwell-synergy.com

Table S1 Association between SNPs in GABAA and GABBR2 and nicotine dependence in a sample of Australian and US adults from the NICSNP Project.

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