Association and Interaction Analyses of GABBR1 and GABBR2 with Nicotine Dependence in European- and African-American Populations

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Abstract

Previous studies have demonstrated that the γ-aminobutyric acid type B (GABA_B) receptor plays an essential role in modulating neurotransmitter release and regulating the activity of ion channels and adenyly cyclase. However, whether the naturally occurring polymorphisms in the two GABA_B receptor subunit genes interact with each other to alter susceptibility to nicotine dependence (ND) remains largely unknown. In this study, we genotyped 5 and 33 single nucleotide polymorphisms (SNPs) for GABA_B receptor subunit 1 and 2 genes (GABBR1, GABBR2), respectively, in a sample of 2037 individuals from 602 nuclear families of African-American (AA) or European-American (EA) origin. We conducted association analyses to determine (1) the association of each subunit gene with ND at both the individual SNP and haplotype levels and (2) the collective effect(s) of SNPs in both GABA_B subunits on the development of ND. Several individual SNPs and haplotypes in GABBR2 were significantly associated with ND in both ethnic samples. Two haplotypes in AAs and one haplotype in EAs showed a protective effect against ND, whilst two other haplotypes in AAs and three haplotypes in EAs showed a risk effect for developing ND. Interestingly, these significant haplotypes were confined to two regions of GABBR2 in the AA and EA samples. Additionally, we found two minor haplotypes in GABBR1 to be positively associated with Heaviness of Smoking Index (HSI) in the EA sample. Finally, we demonstrated the presence of epistasis between GABBR1 and GABBR2 for developing ND. The variants of GABBR1 and GABBR2 are significantly associated with ND, and the involvement of GABBR1 is most likely through its interaction with GABBR2, whereas GABBR2 polymorphisms directly alter susceptibility to ND. Future studies are needed with more dense SNP coverage of GABBR1 and GABBR2 to verify the epistatic effects of the two subunit genes.

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Introduction

Tobacco smoking is a serious public health concern worldwide, as nearly a third of adults smoke tobacco or related products [1]. Nicotine is the main psychoactive substance in cigarettes that functions as a reward and maintains its continued use, ultimately leading to dependence [1]. Meta-analysis of twin and family studies reveals genetic susceptibility to nicotine dependence (ND), with an average heritability of 0.56 [2]. ND also is influenced by environmental factors, as well as by interaction between genetic and environmental factors [2,3].

Substantial efforts have been geared toward identifying genes that predispose individuals to become nicotine dependent. Genome-wide linkage scans of various smoking phenotypes have revealed several regions that likely harbor susceptibility loci for ND [4,3], particularly on chromosomes 9, 10, 11, and 17 [4]. Of these reproducibly identified regions, that on chromosome 9 is of particular interest [6,7,9,10]. The first gene identified from this linkage region was GABBR2 (G-protein coupled receptor 51), for which several SNPs were found to be significantly associated with ND in the Mid-South Tobacco Family (MSTF) cohort [11]. Since that initial report, continued recruitment has yielded approximately a two-fold increase in the sample size of this cohort [10,12].

GABA is the main inhibitory neurotransmitter in the central nervous system, whose actions are mediated by both ionotropic GABA(A) receptors and metabotropic GABA(B) receptors. GABA(B) receptors are seven transmembrane G-protein-coupled proteins that are pharmacologically functional only as a heterodimer consisting of both GABA(A) receptors and metabotropic GABA(B) receptors. GABA(B) receptors are part of the mesolimbic dopamine system, critically important in mediating the reinforcing properties of drugs of abuse. GABA(B) receptors, in particular, are responsible for dampening the reinforcing effects of dopamine resulting from natural reward. Additionally, the GABA system is diffusely expressed in the brain; therefore, areas other than the mesolimbic system may be partly responsible for these effects. Evidence exists...
from both animal and human studies supporting the value of GABA<sub>B</sub> receptor agonists in the treatment of drug abuse. Specifically, in preclinical studies, baclofen, a GABA<sub>B</sub> agonist, has been successful in promoting abstinence and decreasing the use of several drugs of abuse, including nicotine [14]. Baclofen also has been effective in reducing cigarette smoking [15] and has been reported to alter the sensory properties of cigarettes, reducing their desirability [15].

A recent animal study examined the effect of nicotine on GABBR2 expression in various brain regions in a rodent model [16]. After chronic nicotine administration, GABBR2 mRNA was significantly regulated in several brain regions generally associated with addiction, thus providing further evidence that the GABA system is involved in addictive processes. In the mammalian brain, GABA is a major inhibitory neurotransmitter whose modulatory actions are mediated through two types of receptors: the ionotropic GABA<sub>A</sub> and the metabotropic GABA<sub>B</sub> [13]. GABA<sub>A</sub> receptors form ion channels, whereas GABA<sub>B</sub> receptors activate second-messenger systems through G-protein binding and activation. GABA<sub>B</sub> receptors have two subunits, GABAR<sub>B</sub>1 and GABAR<sub>B</sub>2, which must form a heterodimer to be pharmacologically active [17]. Thus, the genes that encode these receptor subunits are of particular interest in the current study.

On the basis of previous human and animal studies suggesting that GABBR2 is involved in the etiology of ND, the current study examined 33 SNPs in GABBR2 in a larger cohort of the MSTF sample. Because the GABAR<sub>B</sub>2 subunit protein does not bind with the neurotransmitter GABA, it must form a heterodimer with the GABA-binding GABAR<sub>B</sub>1 subunit [13]; thus, we also examined the association of five SNPs in GABBR1 with ND.

We found that several SNPs and haplotypes of GABBR2 were significantly associated with ND in both European-American (EA) and African-American (AA) samples. Although we found no evidence for significant associations of GABBR1 with ND in either ethnic sample, we detected a significant gene-gene interactive (epistatic) effect between the two subunits by using a newly developed pedigree-based generalized multifactor dimensionality reduction (PGMDR) approach.

### Materials and Methods

#### Ethics Statement

Informed written consent was obtained in advance from all participants. The study was approved by the Institutional Review Boards of University of Virginia and University of Mississippi Medical Center and was in accordance with the principles of the Helsinki Declaration II.

#### Study Participants

The AA and EA participants were recruited from the US Mid-South States during 1999–2004 [10,12,18]. Detailed information on the clinical characteristics of the samples is given in Table 1 and in previous publications from this group [10,12,18,19]. Proband smokers were required to be at least 21 years old, to have smoked for at least the last five years, and to have consumed at least 20 cigarettes per day for the preceding 12 months. After a smoker proband was identified, we attempted to recruit the biological parents and all full siblings. A total of 2037 participants were included in the current study, with 1366 individuals from 402 AA families and 671 individuals from 200 EA families. The average family size (± standard deviation; SD) was 3.14 ± 0.75 for AA families and 3.17 ± 0.69 for EA families. The average age was 39.4 ± 14.4 years for AA smokers and 40.5 ± 15.5 years for EA smokers. The ND of probands and other smoker participants was assessed with the three measures commonly used in the tobacco research field: Smoking Quantity (SQ; number of cigarettes smoked per day), the Heaviness of Smoking Index (HSI; 0–6 scale), and the Fagerstrom Test for Nicotine Dependence score (FTND; 0–10 scale) as a continuous variable. Given the overlap in the content of the three ND measures, there exist fairly robust correlations among them ($r = 0.68–0.94$) in both the AA and EA samples.

### SNP Selection and Genotyping

A venous blood specimen was obtained from each participant, and genomic DNA was extracted using a Qiagen Maxi kit (Qiagen, Valencia, CA). Five and thirty-three SNPs were selected from the NCBI database (http://www.ncbi.nlm.nih.gov/projects/SNP/; build 120) for the long isof orm of GABBR1 and GABBR2, respectively. The SNPs selected in GABBR2 were intended to provide more uniform coverage with less redundancy of those SNPs genotyped in our previous study [11]. Of the 33 SNPs in GABBR2, only two were genotyped for the second time (i.e., rs2304389 and rs3750344). The SNPs for both genes were chosen on the basis of minor allele frequency ($\geq 0.10$) and to obtain uniform physical coverage of the gene.

The SNPs for GABBR1 were genotyped using a TaqMan assay in a 384-well microplate format, including four no-template negative controls and four positive controls for each homozygous genotyping reaction. Genotyping was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, CA). The GABBR2 SNPs were genotyped using the Illumina BeadChip system at the Center for Inherited Disease Research (CIDR) at Johns Hopkins University.

#### Individual SNP and Haplotype Analysis

First, we used Haplovac (v. 4.0) [20] to identify any inconsistent Mendelian inheritance or other genotyping errors, revealing about a 0.04% error rate for GABBR1 and 0.02% for GABBR2. These samples were excluded from further data analysis. Haplovac (v. 4.0) was then used to assess linkage disequilibrium (LD) and haplotype blocks for all SNPs included in this study. Associations between individual SNPs and three ND measures were determined using the Pedigree-Based Association Test

| Characteristic | African-Americans | European-Americans | Pooled |
|---------------|------------------|-------------------|-------|
| No. of nuclear families | 402 | 200 | 602 |
| Avg. members/family | 3.14 ± 0.75 | 3.17 ± 0.69 | 3.15 ± 0.73 |
| No. of subjects | 1,360 | 671 | 2,037 |
| Female (%) | 66.1 | 69.1 | 67.2 |
| Age (years) | 39.4 ± 14.4 | 40.5 ± 15.5 | 39.7 ± 14.8 |
| No. of smokers | 1,053 | 515 | 1,568 |
| Age of smoking onset | 17.3 ± 4.7 | 15.5 ± 4.4 | 16.7 ± 4.7 |
| Years smoked | 20.4 ± 12.5 | 23.2 ± 13.5 | 21.3 ± 12.9 |
| Smoking quantity/day | 19.4 ± 13.3 | 19.5 ± 13.4 | 19.5 ± 13.3 |
| HSI | 3.7 ± 1.4 | 3.9 ± 1.4 | 3.8 ± 1.4 |
| FTND score | 6.26 ± 2.15 | 6.33 ± 2.22 | 6.29 ± 2.17 |

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Interaction Analysis of GABBR1 and GABBR2

Gene-by-gene interactions between SNPs in GABBR1 and GABBR2 were analyzed using a newly developed PGMDR [24]. Selection of SNPs for interaction analysis for both genes was based on their physical location: SNPs located in exons that encode each functional domain of a subunit protein or SNPs within intronic regions bordered by exons encoding the transmembrane or cytoplasmic domain of each subunit. From GABBR2, we included the following SNPs: rs585819, rs669095, rs6478676, and rs12386336 on either side of exon 13, which encodes a transmembrane protein domain; rs2304389 and rs10985765 in exons 16 and 18, which encode two cytoplasmic protein domains, and rs785648 and rs10818739 in introns 16 and 18. From GABBR1, we included the following SNPs: rs29230 and rs29267 in exon 16 that encodes a transmembrane domain and intron 16, respectively, and rs2267633 in the 3’-UTR and rs2267635 in intron 6, which is bordered by exons that encode the extracellular domain.

As with the previous association analyses, age and sex were included as covariates for the EA and AA samples. Age, sex, and ethnicity were included as covariates in the pooled sample. Gene-by-gene interactions were examined for all two- to seven-locus models. The top-ranked interaction model was chosen for a given order of interaction, and its $P$ value of prediction accuracy (PA) was evaluated by a permutation test based on 1000 shuffles of the adjusted phenotypic values. Because all $P$ values reported here were based on permutation tests of each interaction model, no correction for multiple models is needed. For detailed information on the PGMDR approach, please refer to the paper by Lou et al. [24].

### Results

#### Individual SNP Associations with ND for GABBR1 and GABBR2

Table 2 shows allele frequencies (calculated by directly counting the number of each progenitor allele) and $P$ values for those SNPs that showed significant associations with ND measures in at least one sample before correction for multiple testing. In addition to the seven GABBR2 SNPs identified in our previous report [11], 11 unique SNPs were significantly positively associated with ND in the pooled sample (EA + AA; Table 2). However, the association of individual SNPs varied as a function of ethnicity. That is, each SNP identified as significant in the pooled sample was attributable to either the AA or the EA sample, and there were no SNPs with significant associations shared by the two ethnic groups. Specifically, SNPs rs10120452, rs12337255, rs2900512, rs473221 were associated with measures of ND in the AA sample, whereas SNPs rs2779543, rs6478676, and rs785648 were associated with ND measures in the EA sample. Almost all SNPs were marginally associated with ND measures except for rs785648 ($P = 0.0009$), which remained significantly associated with SQ in the EA sample after correction for multiple testing (Table 2). For GABBR1, analysis revealed no significant association with ND for any individual SNP in the three samples (data not shown); rs29230 exhibited a trend with FTND in the pooled sample ($p = 0.08$).

Using the block definition proposed by Gabriel et al. [25], we revealed eight blocks for GABBR2 in the pooled sample (Figure 1). However, when LDs were assessed separately for each ethnic group, there were slight differences in LD blocks at the 5th end of

| dbSNP ID (allele) | Pooled sample | AA sample | EA sample |
|------------------|--------------|-----------|-----------|
|                  | MAF | SQ | HSI | FTND | MAF | SQ | HSI | FTND | MAF | SQ | HSI | FTND |
| rs1930135 (C/T)  | 0.43 | 0.09a | 0.06a | 0.04a | 0.48 | 0.24a | 0.15a | 0.07a | 0.30 | 0.11a | 0.12r | 0.23r |
| rs2779543 (G/A)  | 0.33 | 0.04r | 0.03d,r | 0.02a | 0.38 | 0.12r | 0.12r | 0.06a | 0.21 | 0.04d | 0.02d | 0.03d |
| rs10120452 (G/A) | 0.26 | 0.008a | 0.02a | 0.01a | 0.30 | 0.03a | 0.05a | 0.03a | 0.17 | 0.07a | 0.07a | 0.07a |
| rs11788000 (T/C) | 0.41 | 0.06a | 0.06a | 0.04a | 0.44 | 0.18r | 0.21r | 0.08r | 0.34 | 0.06a | 0.05a | 0.10a |
| rs12337255 (T/C) | 0.16 | 0.07d | 0.07d | 0.04d | 0.14 | 0.05d | 0.03d | 0.03d | 0.23 | 0.46r | 0.64r | 0.73d,r |
| rs2900512 (C/T)  | 0.26 | 0.03d,r | 0.01d,r | 0.006d,r | 0.28 | 0.06d | 0.02d | 0.01d,r | 0.21 | 0.32d | 0.26d | 0.29d |
| rs7044793 (G/T)  | 0.36 | 0.03a | 0.03a | 0.03d,r | 0.36 | 0.07a | 0.09r | 0.12r | 0.37 | 0.24r | 0.12a | 0.10a |
| rs13295101 (T/C) | 0.36 | 0.04d,r | 0.03d,r | 0.03d,r | 0.36 | 0.08a | 0.08r | 0.11r | 0.37 | 0.28r | 0.16r | 0.13r |
| rs4743221 (A/C)  | 0.28 | 0.06r | 0.08r | 0.02d,r | 0.30 | 0.06r | 0.04r | 0.007d,r | 0.23 | 0.81d | 0.59r | 0.56r |
| rs6478676 (G/A)  | 0.35 | 0.02d,r | 0.03r | 0.03r | 0.32 | 0.22d | 0.56d | 0.40r | 0.43 | 0.008d,r | 0.006d,r | 0.006d,r |
| rs7856488 (C/T)  | 0.34 | 0.006d | 0.05r | 0.04r | 0.23 | 0.60r | 0.89a | 0.82d | 0.37 | 0.0009r | 0.01d,r | 0.005r |

*Corrected $P$ value at the 0.05 significance level is 0.002.

Values significant before correction are shown in bold. Values that survive correction are shown in bold italics.

a = additive model, d = dominant model, r = recessive model.

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the gene. For example, we found four blocks at the 5' end for AAs (Figure 2), and three blocks in the same region for EAs (Figure 3). Specifically, a 34-kb block in EAs containing SNPs rs157927-rs10512258-rs337526-rs337552 existed as two smaller blocks in AAs (rs157927-rs10512258-rs337526, 24 kb, and rs337552-rs509747, 11 kb).

Two blocks were identified in the pooled sample for GABBR1 (Figure 4A); however, when parsed into different ethnic groups, the block structure became different in the two samples. The AA sample had two blocks spanning the whole gene, with the first 4-kb block containing rs2267633-rs29267 and a 17-kb block containing rs29230-rs2267635-rs17184416 (Figure 4B). Conversely, the EA sample had one 21-kb block consisting of SNPs rs2267633-rs29267-rs29230-rs2267635 located at the 3' end of GABBR1 (Figure 4C).

**Haplotype Associations with ND**

Haplotype-based association analyses revealed several major haplotypes that were significantly associated with ND for all sample groups. After correction for multiple testing, there were 14 significant major haplotypes in GABBR2 in the pooled population (Table 3), four in the AA population (Table 4), and five in the EA population (Table 4). All the 14 haplotypes in the pooled sample were located within intronic areas among exons that encode the extracellular, transmembrane, and cytoplasmic domains, with most of them residing among extracellular domain encoding exons 1–9. When the two ethnic groups were analyzed separately, the haplotypes significantly associated with ND phenotypes differed greatly in the AA and EA populations (Table 4). In AAs, all four haplotypes that were significantly associated with FTND and one or two heaviness of smoking measures (HSI and SQ) resided in the intronic areas among extracellular domain-encoding exons (Table 4A and Figure 5A). Conversely, in EAs, four of the five significantly associated haplotypes were located in and around cytoplasmic and transmembrane domain coding exons 16–19 and 10–15, respectively (Tables 4B, 4C and Figures 5B, 5C). Only one haplotype located in the intronic areas among extracellular domain encoding exons was significantly associated with SQ but not with FTND. Interestingly, the same three-SNP combination (i.e., rs3750344-rs6478761-rs2900512) constituted a haplotype in AAs with a differing allele combination, which was significantly associated with FTND and also HSI: The A-A-T haplotype (frequency 24.3%) was negatively associated with HSI ($\zeta = -2.88, P = 0.004$) and FTND ($\zeta = -3.20, P = 0.001$) in AAs, whereas a G-C-C haplotype (frequency 7.4%) was positively associated with SQ ($\zeta = 2.68, P = 0.007$) in EAs. Thus, our results clearly indicate that the significant haplotypes are ethnicity-specific, and the SNP loci of these haplotypes are located in very different parts of the gene in...
Another interesting phenomenon is that the direction of the effects of haplotypes was similar in the AA and EA populations if we did not consider the significance of haplotype effects, suggesting that even if the magnitude of their effects is a bit different in the various backgrounds, the allelic basis of ND in both groups is similar. In addition to the differences in haplotype locations in the gene, the directions of haplotype effects on FTND and heaviness of smoking (SQ and HSI) also differed in the two ethnic groups. As shown in Table 4, in AAs, the haplotypes significant under the recessive model were negatively associated with FTND, SQ, and HSI, whilst the two significant haplotypes under the dominant model were positively associated with FTND. Interestingly, these directions were reversed in EAs; all the significant haplotypes under the recessive model were positively associated with FTND, SQ, and HSI, and the dominant haplotype was negatively associated with SQ.

Haplotype analyses of \textit{GABBR1} revealed no major haplotypes associated significantly with ND (data not shown). There were, however, two minor haplotypes in the EA population (frequency <5\%) that were positively associated with HSI after correction for multiple testing: rs2267635-rs29230-rs29267 (C-G-T; frequency 3.6\%; $\chi^2 = 2.7$, $P = 0.007$) and rs29230-rs29267-rs2267633 (C-T-A; frequency 3.8\%; $\chi^2 = 2.7$, $P = 0.007$) (Table 5), both consisting of SNP rs29230, a synonymous SNP in exon 16 encoding a transmembrane domain protein. Although the SNPs selected for this gene were targeted at the \textit{GABBR1} long isoform, these four SNPs also exist in the short isoforms of \textit{GABBR1}.

**Interaction Analysis of \textit{GABBR1} and \textit{GABBR2}**

On the basis of the knowledge obtained from biochemical and pharmacological studies of the GABA\(_B\) receptor, we investigated the epistatic effect of SNPs from the transmembrane and cytoplasmic domains of each GABA\(_B\) subunit protein because interactions among these domains have biological significance (Figure S1). That is, these regions of the receptor proteins are involved in coupling of the subunits for trafficking to the membrane and for pharmacological activity. Our gene-by-gene interaction analysis revealed significant epistatic effects between \textit{GABBR1} and \textit{GABBR2} (Table 6) on ND in the pooled and EA samples and also interactions among SNPs within \textit{GABBR2} in all three samples (Table S1).

Three significant interactions were detected between SNPs in \textit{GABBR1} and \textit{GABBR2}. The same three-SNP combination was detected for HSI and FTND (rs29230-rs7865648-rs585819; $P = 0.001$ and 0.005, respectively), and the addition of a fourth SNP to this combination was detected for FTND (rs29230-rs7865648-rs669095-rs585819; $P = 0.03$). This four-SNP combination was
contributed by the EA population for FTND ($P = 0.02$), whereas the other interactions were detected only in the pooled sample.

The majority of significant SNP interactions were seen within GABBR2 for the pooled, EA, and AA samples (Table S1). Five significant two- to four-SNP interactions were detected in the pooled sample for SQ ($rs10985765$-$rs7865648$, $P < 0.001$; $rs10985765$-$rs7865648$-$rs6478676$, $P = 0.003$; and $rs10818739$-$rs7865648$-$rs6478676$-$rs585819$, $P = 0.004$) and HSI ($rs10985765$-$rs13286336$-$rs669095$-$rs585819$, $P = 0.05$; and $rs10985765$-$rs7865648$-$rs6478676$-$rs669095$, $P = 0.03$). In the AA population, one four-SNP combination, $rs10985765$-$rs13286336$-$rs699095$-$rs585819$, was significantly associated with all three ND measures ($P = 0.01–0.02$). All other significant two- to four-SNP interactions were identified in the EA sample: SQ and FTND: $rs7865648$-$rs585819$, $P < 0.001$; $rs10818739$-$rs7865648$-$rs6478676$-$rs669095$, $P = 0.003$; and $rs10818739$-$rs7865648$-$rs6478676$-$rs585819$, $P = 0.01$; SQ alone: $rs10818739$-$rs585819$, $P = 0.003$; and $rs10818739$-$rs6478676$-$rs585819$, $P = 0.03$ (Table S1).

**Discussion**

The pharmacologically active GABA_B receptors are formed by heterodimerization of GABA_B1 and GABA_B2 subunit proteins encoded by GABBR1 and GABBR2 genes, respectively. The current study examined the association of GABBR1 and GABBR2 polymorphisms with ND individually and according to their interactive effects in European-American and African-American individuals.

First, our findings revealed a significant association of heaviness of smoking with SNP rs7865648 in EAs and significant associations of ND with 14 major haplotypes in GABBR2 in the pooled sample, after correction for multiple testing. When SNP data were analyzed within each ethnic group, we found two protective and two risk haplotypes unique to the AA sample and one protective and four risk haplotypes unique to the EA sample. It was striking that these unique loci were distributed in different regions of the gene in the two races. Only one common SNP combination was significant in the two ethnic samples but with a differing allelic haplotype in each population, EAs possessing a risk haplotype (G-C-C) and AAs having a protective haplotype (A-A-T). The five polymorphisms in GABBR1 studied here showed no significant associations with any of the ND measures; the smallest $P$ value for association of rs29230 with FTND was 0.06 in the pooled sample. Haplotype analyses revealed two minor haplotypes that were significantly associated with HSI but not with FTND in the EA sample.

Second, in consideration of the fact that functional GABA_B receptors consist of both GABA_B1 and GABA_B2 subunits, we
conducted gene-gene interaction analysis of these two subunit genes in affecting ND. Analyses using our newly developed PGMDR method [24] demonstrated a significant interaction between \textit{GABBR2} and \textit{GABBR1} polymorphisms, confirming previous findings of pharmacological studies that showed GABA\textsubscript{B} receptors function as heterodimers of GABA\textsubscript{B1} and GABA\textsubscript{B2} subunits. Together, our results provide first evidence for direct association of ND with \textit{GABBR2} polymorphisms and an indirect less significant association with \textit{GABBR1} polymorphisms.

Our current study has several unique strengths compared with previous studies from our group and others that implicated loci on chromosome 9, including the \textit{GABBR2} gene, in altering susceptibility to ND [4,16]. On the basis of our early linkage findings, in a previous study, we genotyped SNPs in \textit{GABBR2} and found that seven of the 12 SNPs initially identified were significantly associated with ND [11]. In consideration of the relatively scanty coverage of \textit{GABBR2} and the small sample size in our earlier study, we conducted the current study with the goal of extending our previous results. In the previous study, 12 SNPs were genotyped in about half of the participants of the current MSTF sample. Also, we chose SNPs that provided complete coverage of the gene, without consideration of previously genotyped SNPs. Thus, the current study extends the previous findings by: (a) comprehensively covering the gene by genotyping 33 SNPs for \textit{GABBR2}, (b)
increasing our sample size by ~760 participants, (c) including an association analysis of GABBR1 with ND, and (d) examining the interaction between GABBR1 and GABBR2.

The GABAB1 and GABAB2 subunits dimerize by coiling of C-terminal domains of the two proteins following which the subunits are transported to the plasma membrane, where the receptor becomes pharmacologically active [26,27]. Further evidence suggests the involvement of transmembrane domains in the formation of heterodimers when the C-terminal domain is truncated [28]. This is particularly interesting, considering that, in EAs, haplotypes significantly associated with ND reside in the introns among the transmembrane and cytoplasmic-domain-encoding exons of GABBR2. Conversely, all haplotypes implicated in ND of AAs reside in the introns among exons encoding the binding domain of the GABBR2 subunit protein. The function of the binding domain of GABAB2 is unclear, however, as only GABAB2 is responsible for ligand binding in the heterodimer configuration [29]. Therefore, haplotypes that affect different portions of the GABAB2 heterodimer may have functionally different consequences. For example, EAs may possess GABAB2 subunits that are unable to dimerize with the GABAB1 subunit, which consequently cannot traffic to the membrane, resulting in fewer functional receptors. Conversely, the affected GABAB2 binding domain has not been fully characterized, so it is difficult to speculate on the consequences of these polymorphisms.

Although we found significant associations for GABBR2, but not for GABBR1, with ND, significant interactions between these two subunit genes were evident as reported here. Significant interactions were detected between a synonymous SNP in the transmembrane domain of GABBR1 and SNPs located in the intronic regions among exons encoding transmembrane and cytoplasmic domains of GABBR2. These statistical gene-by-gene interactions are biologically relevant, as the subunits interact to form a complete and functional receptor. Thus, the statistical interaction most likely represents the functional properties of these two subunits. Furthermore, we found that the majority of significant interactions exist within the GABBR2 gene, suggesting a stronger association of ND with GABBR2 polymorphisms compared to associations of ND with both GABBR1 polymorphisms and GABBR1-by-GABBR2 interactive effects. However, it should also be noted that the GABBR2 SNPs included in the interaction models are located in the intronic regions among exons encoding transmembrane and cytoplasmic domains of the GABAB2 subunit. Therefore, these polymorphisms do not affect the amino acid sequence of the transmembrane and cytoplasmic subunits unless there is a strong linkage disequilibrium with a causative variant in an exon; nevertheless, it is possible they affect the structure of mature GABAB2 mRNA through alternate splicing, resulting in altered GABAB2 protein subunits. Although such a molecular mechanism is yet to be elaborated, the presence of six alternatively spliced mRNA variants for GABAB2 (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/) strengthens the significance of functional SNPs in GABBR2 intronic regions.

In sum, this study not only confirms our earlier finding that GABBR2 is associated with ND but also demonstrates that an interaction of GABBR1 and GABBR2 alters susceptibility to ND. Our findings also indicated that GABBR2 represents a major contributor to this phenomenon, and its biological and statistical interaction with GABBR1 implies that the latter is indirectly involved in ND. Furthermore, our results showed that the

### Table 3. Haplotype frequencies, and Z- and P-values for association of three-SNP combinations in GABBR2 with three measures of ND in the pooled sample*.

| SNP Combination | Haplotype | % (# Families) | Z-value (P) |
|-----------------|-----------|----------------|------------|
|                 |           |                | SQ          | HSI        | FTND      |
| **(A) Extracellular Domain:** | | | | |
| rs1571927-rs1930135-rs2779543 | A-T-A | 28.8 (82) | -2.40 (0.017)r | -2.78 (0.006)r | -2.77 (0.006)r |
| rs1930135-rs2779543-rs10120452 | C-G-G | 56.7 (241.4) | 2.16 (0.031)a | 2.32 (0.020)a | 2.52 (0.012)a |
| rs2779543-rs10120452-rs11788000 | G-T-T | 57.7 (234.4) | 2.27 (0.023)d | 2.53 (0.011)d | 2.77 (0.006)d |
| rs10120452-rs11788000-rs2779536 | A-A-C | 22.8 (267.4) | -2.69 (0.007)a | -2.63 (0.009)a | -2.62 (0.009)a |
| rs3750344-rs6478761-rs2900512 | A-T-T | 22.9 (53) | -2.38 (0.017)r | -2.96 (0.003)r | -3.18 (0.002)r |
| rs6478761-rs2900512-rs2808523 | A-C-C | 28.2 (220.3) | 1.98 (0.047)d | 2.38 (0.017)d | 2.62 (0.009)d |
| rs7044793-rs13295101-rs2779552 | T-C-G | 21 (246.4) | 2.65 (0.008)a | 2.09 (0.037)a | 1.99 (0.047)a |
| rs1889983-rs4743221-rs7020345 | A-A-A | 58.3 (218) | 2.29 (0.021)d | 2.37 (0.018)d | 2.87 (0.004)d |
| **(B) Transmembrane Domain:** | | | | |
| rs58519-rs669095-rs6478676 | A-A-G | 16.5 (21) | 2.96 (0.003)r | 2.48 (0.013)r | 2.49 (0.013)r |
| **(C) Cytoplasmic Domain:** | | | | |
| rs2304389-rs7865648-rs10985765 | C-T-T | 33 (84) | 2.65 (0.008)r | 1.94 (0.053)r | 1.90 (0.058)r |
| rs7865648-rs10985765-rs10818739 | T-T-A | 32.7 (83) | 2.88 (0.004)r | 2.16 (0.031)r | 2.17 (0.030)r |

*Values shown in bold are significant after correction for multiple testing of major haplotypes (i.e., haplotypes with a frequency >5%). Corrected P values differ by population and haplotype. Models are the same as in Table 2.

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Table 4. Haplotype frequencies, and Z- and P-values for association of three-SNP combinations in GABBR2 with three measures of ND in the AA and EA populations*.

| SNP Combination                  | Haplotype          | AA sample | Z value (P) | EA sample | Z value (P) |
|----------------------------------|--------------------|-----------|-------------|-----------|-------------|
|                                  |                    | % (# Families) | SQ | HSI | FTND | % (# Families) | SQ | HSI | FTND |
| (A) Extracellular Domain:        |                    |            |   |     |     |            |   |     |     |
| rs1571927-rs1930135-rs2779543    | A-T-A              | 36.4(78)  | -1.93(0.054)r | -2.26(0.024)r | -2.38(0.018)r | 14.1(53.8)  | -1.55(0.120)a | -1.58(0.115)a | -1.47(0.141)a |
|                                  | C-G-G              | 48.7(239.3) | 1.58(0.113)a | 1.83(0.067)a | 2.23(0.026)a | 71.8(32)   | 1.65(0.098)d  | 1.62(0.105)d  | 1.32(0.185)d  |
|                                  | T-A-A              | 29.5(52)   | -2.12(0.034)r | -2.12(0.034)r | -2.12(0.034)r | 145.5(53)  | -1.75(0.080)a | -1.85(0.064)a | -1.86(0.063)a |
| rs2779543-rs10120452-rs11788000  | G-G-G              | 52.4(194.4)| 1.79(0.073)d  | 2.02(0.043)d  | 2.23(0.026)d  | 71.8(32)   | 1.65(0.098)d  | 1.62(0.105)d  | 1.32(0.185)d  |
|                                  | C-G-C              | 25.3(116.5)| -2.16(0.030)a | -2.05(0.040)a | -2.05(0.040)a | 145.5(53)  | -1.75(0.080)a | -1.91(0.056)a | -1.88(0.060)a |
|                                  | T-A-A              | 29.5(52)   | -2.12(0.034)r | -2.12(0.034)r | -2.12(0.034)r | 145.5(53)  | -1.75(0.080)a | -1.85(0.064)a | -1.86(0.063)a |
| rs10120452-rs11788000-rs2779536  | G-T-A              | 54.2(182.5)| 1.71(0.087)d  | 2.03(0.043)d  | 2.46(0.014)d  | 68.8(4)    | 1.87(0.061)a  | 1.83(0.067)a  | 1.63(0.103)a  |
|                                  | T-A-T              | 24.4(39)   | -2.38(0.018)a | -2.04(0.042)a | -2.03(0.042)a | 14.5(32)   | -1.89(0.059)a | -2.04(0.041)a | -2.03(0.042)a |
| rs3750344-rs6478761-rs2900512    | A-C-A              | 25.3(116.5)| -2.22(0.026)r | -2.88(0.004)r | -3.20(0.001)r | 19.9(14)   | -0.98(0.329)r | -0.96(0.336)r | N/A |
|                                  | G-C-G              | 29.3(116.5)| -0.30(0.789)a | -0.74(0.458)a | -0.99(0.320)a | 27.4(28.2)| 2.68(0.007)d  | 1.93(0.054)d  | 2.07(0.039)d  |
|                                  | C-G-C              | 26.7(116.5)| -0.30(0.789)a | -0.74(0.458)a | -0.99(0.320)a | 27.4(28.2)| 2.68(0.007)d  | 1.93(0.054)d  | 2.07(0.039)d  |
|                                  | A-C-C              | 34.8(175)  | 1.99(0.047)d  | 2.37(0.018)d  | 2.62(0.009)d  | 15(43.4)   | N/A        | 0.46(0.643) | 0.39(0.695) |
|                                  | G-T-A              | 24.4(39)   | -3.30(0.0009)r | -3.25(0.001)r | -3.24(0.001)r | 6.9(32.5)   | -1.04(0.299)d | -0.48(0.634) | -0.51(0.621) |
|                                  | A-T-C              | 24.4(39)   | -3.30(0.0009)r | -3.25(0.001)r | -3.24(0.001)r | 6.9(32.5)   | -1.04(0.299)d | -0.48(0.634) | -0.51(0.621) |
| rs7044793-rs13295101-rs2779552   | T-C-G              | 7.4(165)   | 2.11(0.033)a  | 1.28(0.199)a  | N/A        | 29.5(83.4)  | 1.41(0.159) | 1.66(0.097) | 1.60(0.110) |
|                                  | T-C-A              | 25.3(116.5)| -2.38(0.018)  | -2.04(0.042)  | -2.03(0.042) | 14.5(32)   | -1.89(0.059) | -2.04(0.041) | -2.03(0.042) |
| (B) Transmembrane Domain:        |                    |            |   |     |     |            |   |     |     |
| rs58519-rs669095-rs6478767       | A-A-A              | 11.1(116.4)| 0.22(0.827)a  | 0.27(0.784)a  | N/A        | 26.3(14)   | 2.84(0.005)r  | 2.51(0.012)r  | 2.63(0.009) |
| (C) Cytoplasmic Domain:          |                    |            |   |     |     |            |   |     |     |
| rs2304389-rs7885668-rs10958765   | C-T-T              | 20.5(37)   | 0.22(0.827)a  | 0.27(0.784)a  | N/A        | 26.3(14)   | 2.84(0.005)r  | 2.51(0.012)r  | 2.63(0.009) |
| rs7856848-rs10985765-rs10818739  | T-T-A              | 20.2(14.8) | 0.22(0.827)a  | 0.27(0.784)a  | N/A        | 26.3(14)   | 2.84(0.005)r  | 2.51(0.012)r  | 2.63(0.009) |
|                                  | C-T-A              | 27.2(44)   | 0.22(0.827)a  | 0.27(0.784)a  | N/A        | 26.3(14)   | 2.84(0.005)r  | 2.51(0.012)r  | 2.63(0.009) |

*Values shown in bold are significant after correction for multiple testing of major haplotypes (i.e., haplotypes with a frequency >5%). Corrected P values differ by population and haplotype. Models are the same as in Table 2. doi:10.1371/journal.pone.0007055.t004
genetically determined vulnerability to ND is different in subjects of European and African ancestry. These findings are further supported by studies that demonstrated the regulation of receptor expression after nicotine exposure in animal models, as well as the impact on addictive behaviors in both animals and humans of the GABA<sub>B</sub> agonist balcofen.

Table 5. Haplotype frequencies, and Z- and P-values for association of three-SNP combinations in GABBR1 with three measures of ND in EA population*.

| SNP Combination       | Haplotype | % (# Families) | Z-value (P) | SQ   | HSI  | FTND  |
|-----------------------|-----------|----------------|-------------|------|------|-------|
| rs2267635-rs29230-ns29267 | C-T-T     | 8.3 (55)       | -1.30 (0.192)r | -2.02 (0.044)r | -2.07 (0.039)r |
|                       | C-C-T     | 3.6 (22)       | 2.31 (0.020)a,d | 2.72 (0.007)a,d | 2.40 (0.017)a,d |
| rs29230-ns29267-ns2267633 | T-T-A     | 8.4 (55)       | -1.30 (0.193)r | -2.02 (0.044)r | -2.07 (0.039)r |
|                       | C-T-A     | 3.8 (22)       | 2.31 (0.021)a,d | 2.72 (0.007)a,d | 2.40 (0.017)a |

*Values shown in bold are significant after correction for multiple testing of major haplotypes (i.e., haplotypes with a frequency >5%). Corrected P values differ by population and haplotype. Models are the same as in Table 2.

1Note that significant haplotypes are minor haplotypes (i.e., <5%).

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**Table 6.** Detected interaction models for SNPs in GABBR1 and GABBR2.

| Sample | Gene(s) and SNPs included in interaction model | Pooled | GABBR1: rs29230 | GABBR2: rs7865648-rs585819 | GABBR1: rs29230; GABBR2: rs7865648-rs669095-rs585819 | EA |
|--------|-----------------------------------------------|--------|-----------------|-----------------------------|------------------------------------------------|------|--------|
|        |                                               | HSI/FTND | 0.55            | 0.001                       | FTND                                                       | 0.56 | 0.02   |

**Supporting Information**

**Table S1** Supplementary Table S1

Table S1 Supplementary Figure 1

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**Author Contributions**

Conceived and designed the experiments: MDL JZM TJP. Performed the experiments: MDL CS. Analyzed the data: MDL JM CS GBC JZM XYL. Contributed reagents/materials/analysis tools: MDL JZM TJP. Wrote the paper: MDL JM CS XYL TJP.

**References**

1. USDHHS (2000) Reducing tobacco use: A report of the Surgeon General. Atlanta, Georgia: US Department of Health & Human Services, Center for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion.

2. Li MD, Cheng R, Ma JZ, Swann GE (2003) A meta-analysis of estimated genetic and environmental effects on smoking behavior in male and female adult twins. Addiction 98: 23–31.

3. Sullivan PF, Kendler KS (1999) The genetic epidemiology of smoking. Nicotine Tob Res 1 Suppl 2: S51–57; discussion S69–70.

4. Li MD (2008) Identifying susceptibility loci for nicotine dependence: 2008 update based on recent genome-wide linkage analyses. Hum Genet 123: 119–131.

5. Uhl GR, Liu QR, Naiman D (2002) Substance abuse vulnerability loci: converging genome scanning data. Trends Genet 18: 420–425.

6. Gelernter J, Panhuysen C, Weiss R, Brady K, Poling J, et al. (2007) Genomewide linkage scan for nicotine dependence: identification of a chromosome 5 risk locus. Biol Psychiatry 61: 119–126.

7. Bergen AW, Koreczk JF, Weissbecker KA, Goldstein AM (1999) A genomewide search for loci contributing to smoking and alcoholism. Genet Epidemiol 17 Suppl 1: S55–60.

8. Bierut LJ, Rice JP, Goate A, Hinrichs AL, Saccone NL, et al. (2004) A genomic scan for habitual smoking in families of alcoholics: common and specific genetic factors in substance dependence. Am J Med Genet 124A: 19–27.

9. Li MD, Ma JZ, Cheng R, Dupont RT, Williams NJ, et al. (2003) A genomewide scan to identify loci for smoking rate in the Framingham Heart Study population. BMC Genet 4 Suppl 1: S103.

10. Li MD, Payne TJ, Ma JZ, Lou XY, Zhang D, et al. (2006) A genomewide search finds major susceptibility Loci for nicotine dependence on chromosome 10 in african americans. Am J Hum Genet 78: 745–751.

11. Beuten J, Ma JZ, Payne TJ, Dupont RT, Crews KM, et al. (2005) Single- and Multilocus Allelic Variants within the GABAB Receptor Subunit 2 (GABBR2) Gene Are Significantly Associated with Nicotine Dependence. Am J Hum Genet 76: 839–849.

12. Li MD, Ma JZ, Payne TJ, Lou XY, Zhang D, et al. (2006) Genome-wide linkage scan for nicotine dependence in European Americans and its converging results with African Americans in the Mid-South Tobacco Family sample. Mol Psychiatry 13: 467–416.

13. Bettler B, Kaasmann K, Mosbach J, Gasparrelli M (2004) Molecular structure and physiological functions of GABA(B) receptors. Physiol Rev 84: 835–867.

14. Cousins MS, Roberts DC, de Wit H (2002) GABA(B) receptor agonists for the treatment of drug addiction: a review of recent findings. Drug Alcohol Depend 63: 209–220.

15. Cousins MS, Stamat HM, de Wit H (2001) Effects of a single dose of baclofen on self-reported subjective effects and tobacco smoking. Nicotine Tob Res 3: 125–129.

16. Sun D, Huang W, Hwang YY, Zhang Y, Zhang Q, et al. (2007) Regulation by nicotine of Gpr51 and Ntrk2 expression in various rat brain regions. Neuropsychopharmacology 32: 110–116.

17. Ulrich D, Bettler B (2007) GABA(B) receptors: synaptic functions and mechanisms of diversity. Curr Opin Neurobiol 17: 296–303.

18. Li MD, Sun D, Lou XY, Beuten J, Payne TJ, et al. (2007) Linkage and association studies in African- and Caucasian-American populations demonstrate that SHC3 is a novel susceptibility locus for nicotine dependence. Mol Psychiatry 12: 462–473.

19. Li MD, Beuten J, Ma JZ, Payne TJ, Lou XY, et al. (2005) Ethnic- and gender-specific association of the nicotinic acetylcholine receptor alpha2 subunit gene (CHRNA4) with nicotine dependence. Mol Hum Genet 4: 1211–1219.

20. Barrett JG, Fry B, Lander ES, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21: 263–265.

21. Lange C, Lyon H, DeMeo D, Raby B, Silverman EK, et al. (2003) A new powerful non-parametric two-stage approach for testing multiple phenotypes in family-based association studies. Hum Hered 56: 10–17.

22. Horvath S, Xu X, Lake SL, Silverman EK, Weiss ST, et al. (2004) Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. Genet Epidemiol 26: 61–69.

23. Nyholt DR (2004) A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. Am J Hum Genet 74: 765–769.

24. Lou XY, Chen GB, Yan L, Ma JZ, Mangold JE, et al. (2008) A combinatorial approach to detecting gene-gene and gene-environment interactions in family studies. Am J Hum Genet 83: 457–467.

25. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, et al. (2002) The structure of haplotype blocks in the human genome. Science 296: 223–229.

26. Calver AK, Robbins MJ, Cooze C, Rice SQ, Abbas AE, et al. (2005) The C-terminal domains of the GABAB receptor subunits mediate intracellular trafficking but are not required for receptor signaling. J Neurosci 21: 1203–1210.

27. Marquez-Mirovic M, Jan VN, Jan LY (2000) A trafficking checkpoint controls GABAB receptor heterodimerization. Neuron 27: 97–106.

28. Schwarz DA, Barry G, Eliaof SD, Petroski RE, Conlon PJ, et al. (2000) Characterization of gamma-aminobutyric acid receptor GABAB(1e), a GABA-B1 splice variant encoding a truncated receptor. J Biol Chem 275: 32174–32181.

29. Kniazeff J, Galvez T, Labesse G, Pin JP (2002) No ligand binding in the GB2 splice variant of the GABA(B) receptor beta 2 subunit. J Neurochem 79: 735–7361.

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