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Dimensional Anxiety Mediates Linkage of *GABRA2* Haplotypes With Alcoholism

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Abstract

The GABAA α_2 receptor gene (*GABRA2*) modulates anxiety and stress response. Three recent association studies implicate *GABRA2* in alcoholism, however in these papers both common, opposite-configuration haplotypes in the region distal to intron3 predict risk. We have now replicated the *GABRA2* association with alcoholism in 331 Plains Indian men and women and 461 Finnish Caucasian men. Using a dimensional measure of anxiety, harm avoidance (HA), we also found that the association with alcoholism is mediated, or moderated, by anxiety. Nine SNPs were genotyped revealing two haplotype blocks. Within the previously implicated block 2 region, we identified the two common, opposite-configuration risk haplotypes, A and B. Their frequencies differed markedly in Finns and Plains Indians. In both populations, most block 2 SNPs were significantly associated with alcoholism. The associations were due to increased frequencies of both homozygotes in alcoholics, indicating the possibility of alcoholic subtypes with opposite genotypes. Congruently, there was no significant haplotype association. Using HA as an indicator variable for anxiety, we found haplotype linkage to alcoholism with high and low dimensional anxiety, and to HA itself, in both populations. High HA alcoholics had the highest frequency of the more abundant haplotype (A in Finns, B in Plains Indians); low HA alcoholics had the highest frequency of the less abundant haplotype (B in Finns, A in Plains Indians) (Finns: $P < 0.007$, OR $\alpha 2.1$, Plains Indians: $P < 0.040$, OR $\alpha 1.9$). Non-alcoholics had intermediate frequencies. Our results suggest that within the distal *GABRA2* region is a functional locus or loci that may differ between populations but that alters risk for alcoholism via the mediating action of anxiety.

Keywords

SNPs; polymorphisms; GABAA; American Indian; harm avoidance

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INTRODUCTION

γ -Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the CNS. GABA_A receptors are modulated by various drugs including ethanol, benzodiazepines, and barbiturates. These drugs have similar anxiolytic, sedative-hypnotic, anticonvulsant, motor-incoordinating, and cognitive impairing effects. GABA_A receptors are implicated in the acute and chronic effects of alcohol, including tolerance, dependence, and cross-tolerance to benzodiazepines and barbiturates [reviewed in Davies, 2003; Kumar et al., 2004]. This cross-tolerance, together with the effectiveness of benzodiazepines in treating both anxiety and alcohol withdrawal, suggests that GABA_A receptors may play a key role in vulnerability to alcoholism and anxiety.

GABAergic neurotransmission modulates emotion and response to stress. Acute stress immediately reduces GABA-stimulated chloride influx in the frontal cortex and amygdala [Martijena et al., 2002]. Socially isolated rats exhibit anxious behavior accompanied by increased plasma corticosterone and diminished levels of neurosteroids and brain GABA_A receptor function [Serra et al., 2000]. In male rats, early life stress permanently alters GABA_A receptor subunit expression in the hippocampus such that GABA_A α 2 predominates in the stressed animals whereas GABA_A α 1 predominates in emotionally healthy animals [Hsu et al., 2003]. Adult rats that have been subjected to early life stress also have a more active stress response [Hsu et al., 2003]. The GABA_A α 2 subunit (the *GABRA2* gene) may therefore play an important role in mediating stress and anxiety responses. Further support for this view is that the anxiolytic effects of benzodiazepines appear to be mediated by *GABRA2*; mice with a *GABRA2* knock-in point mutation are insensitive to benzodiazepines' anxiolytic effects [Low et al., 2000].

GABA_A receptors are also modulated by neurosteroids such as progesterone and its metabolites (e.g., allopregnanolone) [Engel and Grant, 2001]. Acute exposure to progesterone or allopregnanolone is anxiolytic but prolonged exposure is anxiogenic [Gulinello and Smith, 2003]. Sex differences might, therefore, be expected in the relationship between GABA neurotransmission and anxiety.

There is evidence for linkage of alcohol dependence to chromosome 4p at the location of the GABA_A gene cluster in American Indians and Caucasians [Long et al., 1998; Zinn-Justin and Abel, 1999]. In addition, the intermediate phenotype of relapse-associated beta EEG power mapped to this region [Porjesz et al., 2002]. A mouse QTL for alcohol withdrawal severity is located at the homologous mouse gene cluster [Buck and Hood, 1998]. The Collaborative Study on the Genetics of Alcoholism (COGA) performed a family-based association study on 2,608 predominantly Caucasian individuals from families with a high density of alcoholism. Individuals were genotyped across the GABA_A chromosome 4 gene cluster. A strong haplotype association with both alcohol dependence and beta EEG power was found in a region of *GABRA2* distal to intron 3 [Edenberg et al., 2004]. In another U.S. Caucasian study [Covault et al., 2004], *GABRA2* haplotype distributions were compared between 446 predominantly male alcoholics and 334 predominantly female non-alcoholics. Haplotype association with alcohol dependence was found in the same region of *GABRA2* and the effect was strongest in the 248 alcoholics who did not have drug dependence or

major depressive episode. Finally, a recent Russian study of 113 male alcoholics and 100 male controls supported the findings of the earlier studies [Lappalainen et al., 2005].

Alcoholism is a complex, heterogeneous disease with a heritability of approximately 50% [Heath et al., 1997; Goldman et al., 2005]. There are likely to be many genes with small to modest effects that influence vulnerability to alcoholism. Environmental stressors are as important as genetic vulnerability; environmental influences on the development of alcoholism will differ across populations, across age groups, and also between the sexes. In order to dissect the complex etiology of alcoholism, it is important to distinguish subgroups of alcoholics that differ in genetic and environmental background. From the foregoing discussion, it would seem that *GABRA2* is a good candidate gene for both alcoholism and anxiety. The aim of our study was to determine the associations between *GABRA2* haplotypes and alcoholism, anxious temperament and pathological anxiety in two ethnically and geographically distinct population isolates: Finnish Caucasian men and Plains American Indian men and women. The Finnish alcoholics were primarily violent, incarcerated offenders with a high prevalence of anti-social personality disorder (ASPD), whereas the Plains Indian alcoholics were derived from the community. Thus these two groups of alcoholics are likely to have different environmental stressors and may differ in genetic vulnerability. In addition, we predicted that there might be sex differences in the relationship between *GABRA2* and anxiety, based on the influence of progesterone and its derivatives on GABA neurotransmission. Finally, we predicted that *GABRA2* haplotype block structure and haplotype frequencies differ across ethnic groups, so we also genotyped and performed haplotype analyses on a sample of non-alcoholic African Americans in addition to our two study populations.

MATERIALS AND METHODS

Participants: Plains American Indians

Ascertainment—Volunteers (135 men and 196 women, total 331) were recruited from a Plains Indian tribe living in rural Oklahoma. Probands were initially ascertained at random from the tribal register, and the families of alcoholic probands were extended. Although most participants derived from one large, multigenerational pedigree, the average sharing of descent was only 0.3%. Written informed consent was obtained according to a human research protocol approved by the human research committee of the National Institute on Alcohol Abuse and Alcoholism (NIAAA), NIH. The protocol and consent forms were also approved by the Plains Indian Tribal Council. The mean ages were; women: 44.2 years, SD α 14.8; men: 41.5 years, SD α 12.9.

Psychiatric diagnoses—Blind-rated DSM-III-R lifetime psychiatric diagnoses [American Psychiatric Association, 1987] were derived from the Schedule for Affective Disorders and Schizophrenia-Lifetime Version [SADS-L; Spitzer and Endicott, 1978]. An additional criterion for alcohol dependence, drinking heavily for a year or more, was incorporated to establish a clear pattern of long-term alcohol use. A clinical social worker (B.A.) experienced in the tribal customs and culture conducted the SADS-L interviews and

obtained a Lifetime Drinking History [Skinner and Sheu, 1982]. The Hollingshead Two Factor Index of Social Position measured socioeconomic status [Hollingshead, 1957].

Nearly all the 186 alcoholics had a diagnosis of dependence; only eight had alcohol abuse. The demographics are given in Table I. In this study, the terms, “alcohol use disorders,” “alcoholism,” and “alcoholics” include DSM-III-R abuse and dependence. It should be noted that in the alcoholics, diagnoses of anxiety disorders were made only if they pre-dated the onset of heavy drinking.

There were 38 individuals with lifetime DSM-III-R anxiety disorders: 84% had phobic disorder, 12% had obsessive-compulsive disorder, and 5% had panic disorder.

Participants: Finnish Caucasians

Ascertainment—The sample from Helsinki, Finland, has been described in detail elsewhere [Lappalainen et al., 1998]. In total, 461 men were genotyped (mean age 33.4 years (SD α 10.6)) and comprised: 152 incarcerated alcoholic criminal offenders, 135 relatives (80 alcoholic), and 174 population controls. Of the alcoholics, 189 had alcohol dependence and 43 had alcohol abuse. The demographics are given in Table I. For the total sample, the average sharing of descent was calculated to be only 0.2%.

Written informed consent was obtained according to human research protocols approved by the human research committees of NIAAA and the National Institute of Mental Health, NIH, of the Department of Psychiatry, University of Helsinki, and of the University of Helsinki Central Hospital, Helsinki, Finland.

Psychiatric diagnoses—Blind-rated DSM-III-R psychiatric diagnoses were derived from the Structured Clinical Interview for DSM-III-R (SCID) [Spitzer et al., 1990], administered by psychiatrists to both alcoholics and controls. Individuals with major psychotic episodes were excluded.

Dimensional Measures of Personality

Tridimensional Personality Questionnaires (TPQ) [Cloninger, 1987] were completed by both Plains Indians and the Finnish Caucasians. The TPQ provides dimensional measures of three heritable components of personality: Harm Avoidance (HA), Novelty Seeking (NS), and Reward Dependence. High NS and HA scores have been associated with alcoholism. NS is a dimensional scale for impulsivity, thrill seeking, and disorderliness. HA is a measure of the tendency to be anxious and fearful. Individuals who have high HA have been categorized as cautious, tense, apprehensive, worriers, fearful, shy, and inhibited [Cloninger, 1987]. The TPQ has been widely validated and HA has been shown to be moderately (40–60%) heritable [Heath et al., 1994]. HA scores were normally distributed in both populations.

African American Genotyping Dataset

This dataset was used to compare haplotype block structure and allele and haplotype frequencies across three ethnic groups. Three hundred twenty-seven African American men

were recruited from diabetic clinics and churches as part of a different study. Their non-alcoholic status was derived from SCID interviews. Participants gave informed consent under a human research protocol approved by the Department of Veteran Affairs, New Jersey Health Care System.

Genotyping

Nine SNPs were selected from the Celera Discovery System database to cover the gene (Table II). Genomic DNA was extracted from lymphoblastoid cell lines and 10 ng was dried down in each well of 384-well plates. Genotyping was performed by 5' exonuclease assays (Taqman®) that combine polymerase chain reaction (PCR) amplification and detection into a single step using fluorogenic probes. The probes were fluorescently labeled either with FAM or with VIC at the 5' end. The 3' label for each probe was TAMRA. Oligonucleotide primer/probe sets were obtained as Assays-on-Demand from Applied Bio Systems (ABI, Foster City, CA). The reaction mixture consisted of 2.5 µl of Taqman Master Mix (ABI), 100 nM for each probe, 900 nM forward and reverse primer each, and 10 ng genomic DNA in a total volume of 5 µl. Amplification was performed with a Gene Amp PCR System 9700 (ABI) using 384-well plates and following the amplification profile: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min, 40 cycles at 92°C for 15 sec, and 60°C for 1 min. After PCR amplification, endpoint fluorescence intensity was measured directly by 7900HT Sequence Detector (ABI) and genotypes were determined by Sequence Detection System (SDS) Software Version 2.0 (ABI). The genotyping amplification rate was 98%. Twenty percent of the DNA samples were randomly re-genotyped; the genotyping error rate was <0.01.

Statistical Analyses

The related individuals from the Finnish sample derive from many small nuclear families whereas the Plains Indians derive from one large, complex multi-generational pedigree. The proportion of genetic identity shared between any two individuals through common descent was calculated for all possible pairs (related and unrelated) in each population using SAGE. The average sharing of descent was only 0.3% in the Plains Indians and 0.2% in the Finnish Caucasians. This is less than the degree of relationship between third cousins, and indicates that most pairs of individuals in these two populations have a very low degree of relationship. Nevertheless, as a check, family-based association analyses using FBAT were performed on the Finnish-related individuals for SNPs 4 and 5. These analyses produced almost identical *P*-values to those we found in our analyses of the total sample. The Plains Indian sample had an insufficient number of nuclear families to provide enough power to perform FBAT. We were, therefore, able to undertake group analyses that assume independence of individuals. The Finnish Caucasians are regarded as a population isolate with little or no admixture and we have verified this with a panel of 204 genomic control SNP loci chosen for population informativeness (data not presented). The average Plains Indian ancestry in our sample was 87% (SD 21%); however the median and modal values were 100%. Any admixture was predominantly with other American Indian tribes. Therefore population stratification is unlikely to be an issue in our analyses. Our study was exploratory in nature, so nominal *P*-values are reported and no adjustments were made for multiple

comparisons. Also, the SNPs are not independent, being physically linked. A two-tailed P -value of <0.05 was considered statistically significant.

Allelic associations and Linkage Disequilibrium (LD) (D') matrices were computed across the nine SNPs using Haploview version 2.04 Software (Whitehead Institute for Biomedical Research, USA). Haplotype blocks were identified. Within each block, haplotypes were generated and haplotype frequencies were estimated using a Bayesian approach implemented with PHASE [Stephens and Donnelly, 2003]. The results from PHASE closely agreed with results from a maximum likelihood method (MLOCUS) implemented via an expectation–maximization (EM) algorithm [Long et al., 1995].

Analysis of variance (ANOVA) was employed to compare HA scores across groups. Allele, genotype, and haplotype frequencies were compared using the χ^2 distribution. Logistic regression analyses were used to generate odds ratios.

RESULTS

Allele frequencies varied considerably across the three ethnically diverse groups (Table II). In all three populations, SNPs 1 and 2 were in strong LD with each other ($D' \approx 1.0$) but not with the remaining SNPs, and SNPs 3 through 9 (downstream from intron 3) were in strong LD with each other ($D' > 0.95$). Thus two haplotype blocks were identified in all three populations (Fig. 1). There were no haplotype associations with alcoholism or anxiety in block 1. Results are presented here for block 2, SNPs 3 through 9.

Six common block 2 haplotypes (frequency > 0.05) accounted for 0.94–0.99 of the observed haplotypes across the three populations (Table II). As expected, African Americans had more haplotype diversity. They had four haplotypes of approximately equal frequency, two of which were not present in the Caucasians or American Indians. Two complementary haplotypes (A and B), with exactly opposite allelic configuration, predominated in the Caucasians and Plains Indians. In addition, haplotype C was unique to Caucasians and haplotype D, differing in only one allele from haplotype A, was almost confined to Plains Indians (Table II).

Alcoholics Have Higher HA Than Non-Alcoholics

HA scores were normally distributed in both samples.

The Finnish alcoholics had significantly higher HA than the non-alcoholics (17.5 ± 6.4 vs. 11.1 ± 5.3 , $F(1,473) = 141.1$, $P < 0.0001$). An ANOVA with gender and diagnosis as predictor variables revealed that the Plains Indians alcoholics also had significantly higher HA than non-alcoholics (13.6 ± 5.9 vs. 12.1 ± 5.7 , $F(1,322) = 8.8$, $P = 0.003$). There was a gender effect; women had higher HA than men ($F(1,322) = 8.2$, $P = 0.005$).

Haplotype Association With HA in Alcoholics, But not Non-Alcoholics

In the Finnish alcoholics, HA scores for haplotypes A (18.0 ± 6.2 , $n = 269$) and D (17.6 ± 6.4 , $n = 14$) were higher than for haplotypes B (16.7 ± 6.5 , $n = 160$) and C (16.0 ± 5.8 , $n = 26$). (HA scores (A + D) vs. (B + C): $F(1,467) = 5.1$, $P = 0.024$). In contrast, there were no

haplotype differences in HA scores in non-alcoholics (haplotypes A: 11.0 ± 5.1 , $n = 249$, B: 11.3 ± 5.6 , $n = 176$, C: 11.9 ± 5.2 , $n = 28$, D: 9.9 ± 3.9 , $n = 15$).

In the Plains Indian male alcoholics, HA scores for haplotypes A (12.0 ± 5.1 , $n = 45$) and D (11.1 ± 5.0 , $n = 18$) were lower than for haplotype B (13.5 ± 5.2 , $n = 119$) (C is nonexistent). (HA scores for (A + D) vs. (B): $F(1,180) = 4.8$, $P = 0.031$). There was no association between haplotypes and HA in male non-alcoholics or in the Plains Indian women.

Single-Locus Analyses

Single locus analyses showed that the genotype distributions of SNPs 4 through 8 were significantly associated with alcoholism in the Finns (χ^2 analyses, 2 df, $P = 0.021$ – 0.029) and showed the same trend in the Plains Indian men (χ^2 analyses, 2 df, SNP 4 $P = 0.097$, SNP 5 $P = 0.063$, SNP 6 $P = 0.122$, SNP 7 $P = 0.111$, SNP 8 $P = 0.288$). Further inspection revealed that these associations were in each case due to increased frequencies of both homozygotes in alcoholics. Across SNPs 4 through 8 in both populations, alcoholics had a significant excess of total homozygosity compared with non-alcoholics (χ^2 analyses, 1 df, Finns $P = 0.006$ – 0.008 ; Plains Indian men: SNP 4 $P = 0.043$, SNP 5 $P = 0.027$, SNP 6 $P = 0.062$, SNP 7 $P = 0.054$, SNP 8 $P = 0.125$). The genotypes of alcoholics were not in Hardy–Weinberg equilibrium, whereas the genotypes of non-alcoholics were in Hardy–Weinberg equilibrium. This outcome suggested to us the existence of alcoholic subtypes with differing allelic associations. Other explanations such as population admixture or genotyping error could be ruled out since the Finnish Caucasians and Plains Indians are both population isolates and the genotyping error rate was $<0.01\%$. Because we had already found a significant haplotype association with HA in alcoholics, we investigated the possibility that alcoholic subtypes could be differentiated by HA scores. We took the mean HA score of the non-alcoholics as representative of the mean HA of the population (11 in Finns, 12 in Plains Indians). Alcoholics were then divided into high HA ($>$ population mean), or low HA ($<$ population mean). It can be seen from Table I that high HA and low HA alcoholics do not differ in demographic characteristics.

High HA and low HA alcoholics: different haplotype distributions—There were significant differences in haplotype distributions between non-alcoholics, high HA alcoholics, and low HA alcoholics (Table III). In Finns, high HA alcoholics had the highest frequency of haplotype A (0.59), non-alcoholics were intermediate (0.53), and low HA alcoholics had the lowest frequency (0.42). Correspondingly, low HA alcoholics had the highest frequency of haplotype B (0.46), non-alcoholics were intermediate (0.38), and high HA alcoholics had the lowest frequency (0.31) ($\chi^2 = 9.1$, 2 df, $P = 0.010$, contingency table: haplotypes A & B $\times$ 3 diagnostic groups (non-alcoholics, high and low HA alcoholics). As can be seen in Table II, high HA and low HA alcoholics combined together did not differ from controls in haplotype frequencies.

In Plains Indian men and women, high HA alcoholics had the highest frequency of haplotype B (0.68), non-alcoholics were intermediate (0.64), and low HA alcoholics had the lowest frequency (0.60). Haplotype A showed no variation, however haplotype D (differing in only one allele from haplotype A) was more abundant in low HA alcoholics ($\chi^2 = 7.2$, 2

df, $P = 0.028$ for contingency table: haplotypes B & D across three diagnostic groups). Analysis by sex indicated that the signal came entirely from the men (Table III). High HA male alcoholics were enriched with haplotype B (0.71) compared with low HA alcoholics (0.56) and non-alcoholics were intermediate (0.63). In contrast, high HA male alcoholics had the lowest frequencies of haplotypes A and D (0.29), non-alcoholics were intermediate (0.38), and low HA alcoholics had the highest frequency (0.43) ($\chi^2 = 5.5$, 2 df, $P = 0.063$ for contingency table: haplotypes B & (A + D) across three diagnostic groups; $\chi^2 = 5.3$, 1 df, $P = 0.021$ for contingency table: haplotypes B & (A + D) across high HA and low HA alcoholics) (Table III). As can be seen in Table II, high HA and low HA alcoholics combined together did not differ from controls in haplotype frequencies.

No difference in haplotype distribution between high and low HA non-alcoholics—Table III shows the haplotype distributions in both alcoholics and non-alcoholics divided into high and low HA groups. As discussed earlier, there are significant differences in the distributions of haplotypes A and B between high and low HA male alcoholics; Finns: $\chi^2 = 7.2$, 1 df, $P = 0.007$, OR = 2.1 (95% CI = 1.2–3.6), Plains Indians: $\chi^2 = 4.2$, 1 df, $P = 0.040$, OR = 1.9 (95% CI = 1.0–3.6). In stark contrast, male non-alcoholics similarly divided into high and low HA groups did not differ in haplotype distribution; Finns: $\chi^2 = 0.1$, 1 df, $P = 0.737$, Plains Indians: $\chi^2 = 0.8$, 1 df, $P = 0.374$.

Single locus analyses of high and low HA individuals—Analyses of the seven SNPs supported the haplotype-based analyses. There were significant differences in genotype distributions across the three groups, and allele frequencies differed significantly between high and low HA alcoholics with non-alcoholics having intermediate values (Table IV). Analysis by sex in the Plains Indians revealed that the significant associations were confined to men (Table IV, Fig. 2).

Association With Anxiety Disorders

The haplotype distributions in Plains Indian individuals with lifetime DSM-III-R anxiety disorders ($n = 76$ haplotypes) and without ($n = 590$ haplotypes) were analyzed. Anxiety disorders were associated with increased frequency of haplotype B (0.76 vs. 0.62) and decreased frequencies of haplotype A (0.20 vs. 0.29) and haplotype D (0.03 vs. 0.08) (contingency table: haplotypes B & (A + D) across the two diagnostic groups; $\chi^2 = 6.2$, 1 df, $P = 0.01$). Subsequent analysis by sex showed that the signal came from women only; women (anxiety disorders $n = 40$, no anxiety disorders $n = 346$) $\chi^2 = 6.2$, 1 df, $P = 0.01$; men (anxiety disorders $n = 36$, no anxiety disorders $n = 244$) $\chi^2 = 0.6$, 1 df, $P = 0.43$).

Subsequent single locus analyses showed that in women, all seven SNPs in haplotype block 2 showed significant association across genotype with anxiety disorders (χ^2 : 6.0–10.4, P : 0.049–0.006, 2 df). Finnish men with anxiety disorders ($n = 86$ haplotypes) and without ($n = 892$ haplotypes) did not differ in haplotype nor SNP distributions.

DISCUSSION

We observed the association of *GABRA2* with alcoholism in two groups of alcoholics: urban Finnish Caucasians and rural Plains American Indians that differ in many aspects including

cultural background and socioeconomic status. In addition, the Finnish Caucasian alcoholics were largely incarcerated violent offenders whereas the Plains Indian alcoholics were recruited from the community. Despite their differences, in both populations and in both sexes, the alcoholics had significantly higher HA, a heritable measure of the tendency to be anxious and fearful. This finding is somewhat counter-intuitive in the Finnish alcoholics, given their high rate of ASPD and elevated TPQ NS scores. However, it is possible that in some individuals, impulsive, aggressive behavior may be a paradoxical response to innate anxiety, as it is in some other mammals such as dogs. Taken together, our findings suggest that increased trait anxiety may be an important vulnerability factor for alcoholism. Nevertheless, it should be mentioned that long-lasting anxiety and dysphoria (allostasis) might be an outcome of alcoholism, even in maintained abstinence (Goldman and Barr, 2002). Only longitudinal studies can determine whether increased anxiety is a pre-morbid risk factor.

The non-alcoholics' genotypes were in Hardy–Weinberg equilibrium. The alcoholics' genotypes were out of Hardy–Weinberg equilibrium due to the excess of both homozygotes, and because of this, as well as the known role of *GABRA2* in anxiety and our observed haplotype association of *GABRA2* with HA, we defined two subgroups of alcoholics: high HA alcoholics, the majority group, who had a more anxious personality than non-alcoholics, and low HA alcoholics who were less anxious than non-alcoholics. We defined high and low HA as being respectively above and below the mean HA of the non-alcoholics in each population rather than a global mean HA score because differing environmental stressors in each population may modify baseline levels of anxiety.

In both the Finns and Plains Indians, there were two common complementary haplotypes, A and B, accounting for 89–92% of chromosomes in non-alcoholics. In both samples, the more abundant haplotype (A in Finns and B in Plains Indians) was higher in frequency in high HA alcoholics and the less abundant haplotype (B in Finns and A in Plains Indians) was higher in frequency in low HA alcoholics. In both samples, non-alcoholics were intermediate in these haplotype frequencies. In contrast, there was no variation in haplotype distribution in non-alcoholics divided into high and low HA groups, indicating that the association of *GABRA2* with alcoholism is not simply due to a haplotype association with HA per se.

At first glance, the association of both complementary haplotypes with high HA alcoholism is puzzling. One possible explanation may be that Caucasian and American Indian alcoholics with high and low HA have opposite characteristics. The data we have presented in Table I do not show clear differences although it hints at possibilities; for example, the proportions for MD and ASPD differ between HA groups in both populations. Larger sample sizes are needed and other characteristics need to be dissected in order to understand this conundrum; for example, patterns of drinking tend to vary between Caucasians, who are often maintenance drinkers, and American Indians, who tend to engage in heavy, episodic bouts of drinking. There are several other possible explanations. There could be different functional loci. Also, different gene–environment interactions may result in the same phenotype. There are other examples of paradoxical allelic associations; for example, alcoholism has been associated with both alleles of the functional serotonin transporter promoter polymorphism, 5-HTTLPR. Also, addictions have been associated with both

alleles of the functional catechol-O-methyltransferase Val158-Met polymorphism [Enoch et al., 2006].

Three earlier studies have found an association with alcoholism in the same *GABRA2* haplotype block that is distal to intron 3 [Covault et al., 2004; Edenberg et al., 2004; Lappalainen et al., 2005]. The two studies in entirely Caucasian populations [Covault et al., 2004; Lappalainen et al., 2005] also identified two common complementary haplotypes in this *GABRA2* block that, as in our study, accounted for 91–93% of chromosomes in non-alcoholics. In our study, we found that the more common of the two complementary haplotypes was present in 56% of Finnish male alcoholics (59% in those with high HA, 42% in those with low HA) and 53% of Finnish male non-alcoholics. Covault et al. [2004] found that the more abundant of the two haplotypes was present in 48% of U.S., predominantly male alcoholics and 56% of U.S., predominantly female, non-alcoholics, and likewise in Lappalainen et al. [2005] the more abundant haplotype was present in 48% of Russian male alcoholics and 54% of Russian male non-alcoholics. Thus the results of our study, if true, would predict that the alcoholics in these two studies have low HA. In the COGA study [Edenberg et al., 2004], the sample was predominantly Caucasian but also included African Americans and Hispanics and this may account for the greater haplotype diversity compared to our and the two earlier Caucasian studies. Edenberg et al. [2004] reported two main haplotypes accounting for 82% of chromosomes; the more abundant haplotype was associated with alcoholism and was present in 50% of U.S. male alcoholics and 53% of U.S. female alcoholics. Although the methodology of the COGA study is not directly comparable with the Covault et al. [2004] and Lappalainen et al. [2005] studies, there is suggestive evidence from these three studies that both of the two common complementary haplotypes can be risk factors for alcoholism in different populations. Our study suggests that dimensional anxiety may be the mediating factor. In addition, we have shown (Table II) that African Americans, even though an admixed population, have far lower frequencies of haplotypes A and B (0.56) compared with Caucasians (0.89) and Plains Indians (0.92) and theoretically this may reduce their risk of developing alcoholism.

Our results indicate that sex should be included as a covariate in a mixed gender sample; we found sexual dimorphic effects in the Plains Indians and it is possible that this effect may extend to other communities. The influence of progesterone and its metabolites on GABA neurotransmission may possibly have some bearing on these sex differences. Although there is no sex difference in the heritability of alcoholism, the genes that are involved in alcoholism vulnerability appear to overlap only partially in men and women [Prescott et al., 1999]. In addition, environmental stressors influencing anxiety and excessive alcohol consumption may differ between the sexes and may vary across populations.

There are caveats to our study. Neither population was totally unrelated and this may have introduced some bias, although the degree of relatedness between any two individuals was less than that between third cousins. We have shown the existence of considerable variability in *GABRA2* allele and haplotype frequencies across three different ethnicities, indicating that population stratification could be an issue. Although we studied two populations that are essentially isolates, population stratification might still have an effect on our results.

The advantage of haplotype analysis is that disease associations can be narrowed down to regions of LD, negating the need to genotype every SNP across the gene. Haplotype analyses do not identify functional loci. As yet, the location of a *GABRA2* functional polymorphism(s) is not known. Our study, together with earlier studies, has shown that the association with alcoholism and anxiety is within a haplotype block that extends downstream from intron 3 and not with the proximal haplotype block that includes the promoter region. Recent studies have shown that there are complex patterns of *GABRA2* transcription involving alternative splicing and alternative promoters and at least four major isoforms have been identified in several brain regions that have alternative 5' and 3' regions [Jin et al., 2004; Tian et al., 2005]. Thus identifying functionality in this gene is proving to be a complex undertaking.

In conclusion, the results of our study extend earlier findings of a relationship between genetic variation in *GABRA2* and alcoholism by demonstrating a complex interaction with anxiety. In addition, we have found that effects may vary between ethnic groups and between men and women. We have shown that by breaking down the alcoholism phenotype into subtypes, more detailed genetic information may be obtained from smaller datasets. A major strength of our study is that we have found the same results in two very different populations, nevertheless our results should be treated with caution until replicated in similar datasets because this is in essence an exploratory study with an increased risk of false positive results.

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FIGURE 1.

GABRA2 linkage disequilibrium (LD) in Finnish Caucasians and Plains Indians.

Pairwise LD was computed using Haploview. D' values of 1 are represented by blank squares. Haplotype block boundaries are defined using a minimum average D' value of 0.80. A schematic of the gene is given in the middle of the figure with the positions of the 9 SNPs indicated by lines. The 10 exons are represented by rectangles.

FIGURE 2.

GABRA2 SNP 5 showing increased frequency of both homozygotes (A1A1 and A2A2) in male, but not female, alcoholics

Finns: A1A1 is increased in high HA alcoholics, A2A2 is increased in low HA alcoholics. In contrast, in Plains Indian men A1A1 is increased in low HA alcoholics whereas A2A2 is increased in high HA alcoholics. Plains Indian women show no differences.

High HA defined as mean HA of non-alcoholics; Low HA defined as < mean HA of non-alcoholics

TABLE 1

Demographic Data: Plains Indians

	MEN			WOMEN		
	Non-Alcoholics	Alcoholics High HA	Alcoholics Low HA	Non-Alcoholics	Alcoholics High HA	Alcoholics Low HA
N	38	59	38	107	60	29
Age (yrs)	39.9 ± 16.6	43.8 ± 10.4	39.5 ± 12.0	44.7 ± 16.7	44.2 ± 11.9	42.2 ± 12.7
Yrs Education	12.4 ± 1.6	11.2 ± 1.6	12.7 ± 1.6 ^a	12.0 ± 1.9	11.5 ± 1.8	11.9 ± 1.6
Hollingshead	29.1 ± 11.8	29.6 ± 10.3	32.3 ± 11.0	29.5 ± 12.9	26.8 ± 11.8	27.9 ± 9.6
HA	10.1 ± 5.0	16.3 ± 3.5	7.7 ± 2.9	12.9 ± 5.8	17.7 ± 4.8	7.4 ± 2.3
NS	14.1 ± 4.6	14.9 ± 4.6	14.4 ± 4.3	12.6 ± 4.0	13.9 ± 3.9	12.4 ± 3.8
% smokers	49	64	60	32	40	43
% ASPD	0	26	30	1	6	19
% MD	3	14	14	7	30	24
% anx dis	0	20	13	6	20	17
% currently drinking		39	39		33	31
Onset heavy drinking (yrs)		19.9 ± 5.8	19.7 ± 5.2		22.4 ± 7.2	20.0 ± 5.3
drinks / day		13.6 ± 8.7	12.9 ± 6.8		10.3 ± 5.8	11.4 ± 6.1
Max drinking days / month		11.7 ± 8.5	11.5 ± 7.5		9.0 ± 5.7	9.3 ± 8.4
Yrs heavy drinking		18.7 ± 8.9	14.3 ± 9.7		14.2 ± 11.2	15.1 ± 11.4

^a χ^2 comparisons between high HA and low HA alcoholics;^a $p < 0.0005$

Tridimensional Personality Questionnaire; HA: harm avoidance, NS: novelty seeking

High HA defined as mean of non-alcoholics, i.e. 12

Low HA defined as < mean of non-alcoholics, i.e. <12

MD: DSM-III-R lifetime major depression; anx dis: DSM-III-R lifetime anxiety disorders; ASPD: DSM-III-R lifetime antisocial personality disorder

Hollingshead index of socioeconomic status

Drinks/day: mean drinks per drinking day

TABLE 2**Demographic Data: Finnish Caucasian Men**

	Non-Alcoholics	High HA Alcoholics	Low HA Alcoholics
N	229	198	34
Age (yrs)	33.7 ± 9.1	35.8 ± 14.4	32.8 ± 11.2
Age onset heavy drinking (yrs)		20.8 ± 5.9	22.3 ± 9.0
Harm avoidance	11.1 ± 5.3	19.1 ± 5.1	7.6 ± 2.1
Novelty seeking	15.7 ± 4.2	19.0 ± 4.5	18.1 ± 5.2
% with ASPD	0	46	44
% with MD	3	13	6
% with anxiety disorders	7	12	3

Tridimensional Personality Questionnaire; harm avoidance (HA), novelty seeking

High HA defined as mean of non-alcoholics, i.e. 11

Low HA defined as < mean of non-alcoholics, i.e. <11

DSM-III-R lifetime diagnoses: major depression (MD), anxiety disorders and antisocial personality disorder (ASPD)

TABLE 3
GABRA2 SNP Allele Frequencies in Non-Alcoholics; Differences across 3 Ethnic Groups

SNPs	Marker ^a	Base change	Finnish Caucasians N = 229	Plains Indians N = 145	African Americans N = 327
1	rs2119767	T>A	0.32	0.60	0.17
2	rs9291283	G>A	0.22	0.18	0.28
3	rs10805145	T>C	0.46	0.65	0.42
4	rs279858	T>C	0.41	0.65	0.27
5	rs279863	C>A	0.41	0.65	0.27
6	rs548956	T>C	0.59	0.36	0.74
7	rs529826	T>C	0.42	0.64	0.73
8	rs496650	C>A	0.59	0.36	0.25
9	rs573400	T>C	0.39	0.69	0.26

SNP: single nucleotide polymorphism

^a markers are identified by their rs numbers from the dbSNP database

The African American data is provided solely to compare allele frequencies across 3 ethnicities.

TABLE 4

GABRA2 Block 2 Haplotype Frequencies; Differences across 3 Ethnic Groups

Haplotypes	Finnish Non-Alcs	Caucasians Alcoholics	Plains Non-Alcs	Indians Alcoholics	Af Americans Non-Alcs
N	458	464	290	372	654
A 1112121	0.52	0.56	0.30	0.26	0.29
B 2221212	0.37	0.35	0.62	0.64	0.27
C 2112121	0.06	0.05			
D 1112122	0.01	0.01	0.05	0.09	
E 1112211					0.20
F 2112211					0.18

The African American data is provided solely to compare haplotype frequencies across 3 ethnicities.

TABLE 5
GABRA2 Haplotype Frequencies in Non-Alcoholics and Alcoholics Divided into High and Low Harm Avoidance (HA) Groups

Finnish Men		Non-Alcoholics		Non-Alcoholics		Alcoholics	
Haplotypes		HA	11	HA < 11	HA	11	HA < 11
A	1112121	0.53		0.52	0.59		0.42
B	2221212	0.39		0.36	0.31		0.46
C	2112121	0.06		0.07	0.06		0.04
D	1112122			0.01	0.01		
N		246		212	396		68
Plains Indian Men		Non-Alcoholics		Non-Alcoholics		Alcoholics	
Haplotypes		HA	12	HA < 12	HA	12	HA < 12
A	1112121	0.24		0.34	0.23		0.28
B	2221212	0.69		0.59	0.71		0.56
C	2112121						
D	1112122	0.07		0.07	0.06		0.15
N		32		44	118		76
Plains Indian Women		Non-Alcoholics		Non-Alcoholics		Alcoholics	
Haplotypes		HA	12	HA < 12	HA	12	HA < 12
A	1112121	0.32		0.28	0.28		0.27
B	2221212	0.64		0.66	0.64		0.64
C	2112121						
D	1112122	0.04		0.05	0.08		0.09
N		109		105	120		58

High HA defined as mean HA of non-alcoholics, i.e. 12 (Plains Indians), 11 (Finns)

Low HA defined as < mean HA of non-alcoholics, i.e. <12 (Plains Indians), <11 (Finns)

GABRA2 single nucleotide polymorphisms (A1, A2 alleles) in haplotype block 2. Differences in A2 allele frequencies and genotype distributions between non-alcoholics and alcoholics divided into high and low harm avoidance (HA) groups

TABLE 6

		Alcoholics Low HA		Non-Alcoholics	Alcoholics High HA				
SNP		A2	Allele	Frequency	χ^2	p (2df)	Genotype χ^2	Distrib p (4df)	
Finnish Caucasian Men	3	0.55	0.47	0.40	6.7	0.035	12.3	0.015	
	4	0.48	0.41	0.35	6.5	0.039	13.1	0.011	
	5	0.50	0.42	0.35	7.7	0.023	14.3	0.006	
	6	0.49	0.59	0.65	8.8	0.012	14.6	0.006	
	7	0.53	0.42	0.34	10.5	0.005	15.2	0.004	
	8	0.47	0.58	0.60	9.8	0.008	15.4	0.004	
	9	0.49	0.39	0.33	7.9	0.019	8.5	0.076	
	N	34	229	198					
SNP		A2	Allele	Frequency	χ^2	p (2df)	Genotype χ^2	Distrib p (4df)	
Plains Indian Men	3	0.55	0.64	0.70	4.6	0.010	15.5	0.004	
	4	0.55	0.64	0.72	5.4	0.068	17.8	0.001	
	5	0.54	0.64	0.73	7.4	0.024	20.3	<0.001	
	6	0.42	0.36	0.30	4.0	0.139	13.3	0.010	
	7	0.54	0.64	0.71	5.7	0.059	17.7	0.001	
	8	0.46	0.38	0.28	5.9	0.053	13.5	0.009	
	9	0.71	0.70	0.75	0.8	0.689	7.1	0.129	
	N	38	38	59					
SNP		A2	Allele	Frequency	χ^2	p (2df)	Genotype χ^2	Distrib p (4df)	
Plains Indian Women	3	0.61	0.65	0.65	0.4	0.814	1.3	0.860	
	4	0.59	0.65	0.65	0.7	0.722	1.8	0.764	
	5	0.61	0.66	0.65	0.6	0.760	2.1	0.717	
	6	0.37	0.36	0.35	0.1	0.946	0.8	0.942	
	7	0.62	0.64	0.65	0.1	0.930	1.9	0.745	
	8	0.38	0.36	0.35	0.1	0.937	1.6	0.816	
	9	0.72	0.69	0.72	0.6	0.750	2.5	0.637	

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