Published in final edited form as:

Addict Biol. 2013 March ; 18(2): 357–369. doi:10.1111/j.1369-1600.2012.00457.x.

GABRA2 MARKERS MODERATE THE SUBJECTIVE EFFECTS OF ALCOHOL

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Abstract

Background—Individual differences in subjective responses to alcohol (SR) are moderated by genetic variants and may be risk factors for the development of alcohol use disorders. Variation in the GABA_A α2 receptor subunit gene (*GABRA2*) has been associated with alcohol dependence (AD). Therefore, we examined whether individual differences in SR, which reflect sensitivity to the effects of alcohol, are associated with variation in *GABRA2*.

Methods—Sixty-nine healthy subjects (21–30 yr) underwent a laboratory-based within-session, cumulative oral alcohol dosing procedure, achieving a mean peak blood alcohol level of 100.4 mg/dL (SE = 2.5). Subjective assessments were obtained throughout the session, including ascending and descending limbs of the alcohol curve. We genotyped single nucleotide polymorphisms (SNPs) across the chromosome 4 region spanning *GABRA2* and analyzed the effect of genotype and haplotypes on subjective responses to alcohol. Population substructure was characterized through the use of ancestry informative markers.

Results—Individual SNP analysis demonstrated that carriers of the minor alleles for SNPs rs279858, rs279844, rs279845, rs279826, rs279828 and rs279836 had lower “Negative” alcohol effects scores than individuals homozygous for the common allele at each SNP (p=0.0060, p=0.0035, p=0.0045, p=0.0043, p=0.0037, p=0.0061, respectively). Haplotype effects of block 1 showed concordant results with SNPs in this block (p=0.0492 and p=0.0150 for haplotypes 1 and 4, respectively). The minor alleles for several of these SNPs have previously been associated with AD.

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Authors Contributions

MU, EMW, MEM, HRK and GSW were responsible for the study concept and design, data acquisition, and interpretation of findings. XG, XY and NL assisted with data analysis and interpretation of findings. MU drafted the manuscript. EMW, MEM, HRK and GSW provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

Conclusions—Our findings provide further evidence that variation within *GABRA2* is associated with attenuated negative responses to alcohol, a known risk factor for vulnerability to alcohol use disorders.

Keywords

Alcoholism; Alcohol Dependence; GABA; *GABRA2*; Subjective Response

Introduction

Risk of AD has a substantial genetic component, with heritability of 0.52–0.64 (Bienvenu et al., 2011; Kendler, 2001). GABA (gamma-aminobutyric acid) is the most abundant and widespread inhibitory neurotransmitter in the central nervous system. The actions of GABA are mediated by receptors belonging to two major classes, termed GABA_A and GABA_B (Chu et al., 1990). Specifically, GABA_A receptors, which are coupled with chloride channels, have been suggested to contribute to ethanol's actions (Chu et al., 1990; Korpi et al., 2007). Functional mammalian GABA_A receptors are formed by the assembly of five subunit proteins, with the usual subunit arrangement of two alpha, two beta, and one gamma or delta subunit (Olsen et al., 2007; Tretter et al., 1997). GABA_A receptors are the principal site of action of benzodiazepines (Mohler et al., 2002). These receptors also mediate several behavioral effects of alcohol, and the molecular mechanisms mediating these effects are under current investigation (Korpi et al., 2007; Olsen et al., 2007).

Two genome-wide scans provided evidence for linkage of AD to chromosome 4p (Long et al., 1998; Reich et al., 1998). By fine mapping this region, using family-based association methods, the COGA research group (Edenberg et al., 2004) found that SNPs and their haplotypes throughout the gene encoding the GABA_A $\alpha 2$ subunit (*GABRA2*) were associated with AD. This finding has subsequently been replicated using haplotypic association in different case-control samples (Covault et al., 2004; Enoch et al., 2009; Enoch et al., 2006; Fehr et al., 2006; Lappalainen et al., 2005; Soyka et al., 2008). The significance of these findings is underscored by animal studies, which identify the $\alpha 2$ subunit as the primary alpha subunit in limbic regions (Fritschy and Mohler, 1995), and a key mediator of the anxiolytic effects of benzodiazepines (Low et al., 2000). However, results of human studies remain contradictory, as demonstrated in a recent report in which no evidence of an association between 3'-*GABRA2* polymorphisms and alcoholism was observed in an Italian sample (Onori et al., 2010).

Therefore, the use of endophenotypes, or specialized intermediate phenotypes, has been proposed as a strategy to aid gene identification efforts for complex phenotypes, such as AD (Gottesman and Gould, 2003). Endophenotypes are rigorously defined (Ray et al., 2010) and allow for a critical analysis of genetic risk for alcohol use disorders (AUDs). As part of the COGA project, the use of endophenotypes has successfully led to the identification of genes associated with AD (Dick et al., 2006). Of note, Edenberg et al (Edenberg et al., 2004) reported that variation at the *GABRA2* locus is associated with EEG β power. Another potential endophenotype is subjective response to alcohol, a measure of individual differences in sensitivity to the pharmacological effects of alcohol, and a predictor of the development of AUDs (Morean and Corbin, 2010; Quinn and Fromme, 2011). The exact pattern of subjective responses to alcohol associated with increased risk for alcohol problems, however, remains unclear. In the Low Level of Response (LLR) Model, high-risk individuals experience a dampened response to the full range of alcohol effects; thus a LLR to alcohol appears to be an inherited intermediate phenotype for the development of AUDs (Heath et al., 1999; Schuckit and Smith, 1996, 2000; Trim et al., 2009). However, the Differentiator Model (DM) asserts that high risk status is associated with a greater response

to alcohol's positive, stimulant effects, which are most prominent on the ascending limb of the blood alcohol concentration (BAC) curve, and a lower response to negative, sedative effects, which are most prominent on the descending limb of the BAC curve (Newlin and Thomson, 1990).

Given that twin studies have shown that inherited factors account for 40% to 60% of the variance in alcohol sensitivity (Heath et al., 1999), and under the hypothesis that the same genes that predict increased risk for alcoholism may also mediate a distinctive response to alcohol, studies have begun to examine the association of subjective responses to alcohol with variants in *GABRA2*. In one such study, individuals homozygous for the more common allele of a *GABRA2* single SNP (rs279858) reported greater subjective effects of alcohol than individuals with 1 or 2 copies of the AD-associated minor allele (Pierucci-Lagha et al., 2005). However, this study examined only a single SNP. More recently, in a study that used the alcohol clamp method for intravenous (i.v.) alcohol administration, subjects with the more common allele in 3 SNPs located in the middle of *GABRA2* showed greater subjective responses to alcohol than individuals homozygous for the AD-associated minor alleles (Roh et al., 2010).

The present study examined the effects of genetic variation within the *GABRA2* gene region on the subjective effects of alcohol in healthy social drinkers. Differing from previous studies that used an oral challenge, we evaluated the effects of several SNPs within the region and their haplotypes on the subjective responses to alcohol. In addition, as opposed to the i.v. paradigm, we used a within-session, cumulative oral alcohol dosing procedure that more closely approximates real-world alcohol consumption (Morean and Corbin, 2010; Quinn and Fromme, 2011). Based on the previous studies, we hypothesized that individuals with *GABRA2* variants shown to be associated with AD would have lower subjective responses to alcohol.

Subjects and Methods

Subjects

Sixty-nine healthy subjects between the ages of 21 and 30 years were recruited to participate by newspaper advertisements and posted flyers in the Baltimore area. Subjects who appeared to qualify for research participation based on a telephone screen were invited for an in-person interview. All participants provided written informed consent approved by the Johns Hopkins Medicine Institutional Review Board. Subject assessment included a medical history and physical exam, complete blood count, comprehensive metabolic panel (including renal and hepatic function tests), electrocardiogram, urinalysis, alcohol breathalyzer test and urine toxicology screen. The Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) (Bucholz et al., 1994) was administered by a master's degree-level interviewer to identify DSM-IV axis I psychiatric diagnoses, including past or current diagnoses of alcohol and drug abuse or dependence. Minimum and maximum alcohol consumption patterns were established for this study, quantified using the 90-day Time Line Follow Back (TLFB) (Sobell et al., 1988). To minimize adverse reactions to the alcohol administration, participants had to report at least two drinking episodes in any 30-day period during the 90 day TLFB and at least one of these episodes had to entail two or more drinks for women and three or more drinks for men. In addition, subjects could not report drinking more than 28 alcoholic drinks per month for women or 56 alcoholic drinks for men (i.e., less than NIAAA guidelines for heavy/hazardous drinking). Subjects with heavy/hazardous drinking or current alcohol use disorders were excluded because of the potential for such subjects to have already undergone a change in alcohol sensitivity due to heavy alcohol exposure.

Additional exclusion criteria were: (a) serious medical conditions or use of prescription medications, (b) current or lifetime history of a DSM-IV axis I disorder, (c) use of psychoactive medications within the past 90 days, (d) treatment in the last 6 months with any medication that may affect GABAergic function, including antidepressants, antipsychotics, sedative hypnotics, glucocorticoids, appetite suppressants, opioids, or dopaminergic medications, (e) positive urine toxicology screen, or (f) positive pregnancy test or lactating.

Laboratory Procedures

Alcohol dosing—A terminal blood ethanol concentration of 100mg/dL was selected based on our experience in previous studies (McCaul et al., 2000; Turkkan et al., 1988). The total alcohol dose needed to achieve a BAC of 100 mg/dL was divided equally over three drinks. Each alcohol drink was prepared by mixing the appropriate amount of 95% ethyl alcohol (Spectrum Chemicals) with a non-calorically sweetened beverage (Kool-Aid) to achieve a volume of 4 oz. Placebo drinks were prepared by floating one ml of ethanol on top of the 4 oz of sweetened beverage immediately prior to administration to blind the subject to its content. Also as part of the blinding procedures, an ethanol-soaked wrist band was placed around each glass to deliver a strong alcohol odor.

Alcohol Challenge Session—On the session day, subjects completed a urine toxicology screen, alcohol breathalyzer test, and urine pregnancy test; positive finding/s resulted in termination of their study participation. Subjects reported to the laboratory fasting and then consumed a calorie-restricted meal to control for possible dietary effects on alcohol absorption. Subjects were in a quiet laboratory room, where they were monitored by the research nurse. A cumulative dosing procedure was used. At time 0, a placebo drink was administered. Three equal doses of alcoholized beverage were then administered at 45 min intervals (+45, +90 and +135); subjects had 10 minutes to finish each drink. Subjects completed subjective response assessments using a laptop computer and mouse at baseline (–25 min), following each drink (+15, +60, +105, +150 min) and at 45-minute intervals for the remainder of the session (+195, +240, +285 min) (Figure 1). Following completion of the laboratory session, subjects were admitted to the inpatient Johns Hopkins General Clinical Research Unit to monitor them while intoxicated and were discharged the following morning.

Subjective Response Assessments—*Visual Analog Drug Effect Questionnaire* (VAS) includes the following questions 1) “Do you feel GOOD effects from the drink(s)?”; 2) “Do you feel BAD effects of the drink(s)?”; 3) “Do you LIKE the effects of the drink(s)?”; 4) “Do you DISLIKE the effects of the drink(s)?”; 5) “Is this the WORST you have ever felt?”, and 6) “Is this the BEST you have ever felt?”. Subjects responded by positioning an arrow on a 100-point line anchored by “not at all” on the left and “extremely” on the right. These measures were summed to yield two scales: (a) “Positive” effects, which is the sum of “Good, Like and Best” effect responses, and (b) “Negative” effects, which is the sum of “Bad, Dislike and Worst” effects responses.

Biphasic Alcohol Effects Scale (BAES) (Martin et al., 1993) is composed of 14 items measuring both stimulant and sedative effects of alcohol. Subjects rated the extent to which they experienced these feelings at the present time on a scale from 1 (not at all) to 9 (extremely). These measures were summed to yield two scales: (a) “Stimulant” effects, which are the sum of “elated, energized, excited, stimulated, talkative, up, and vigorous” effects, and (b) “Sedative” effects, which are the sum of “difficulty concentrating, down, heavy head, inactive, sedated, slow thoughts and sluggish” effects.

Subjective High Assessment Scale (SHAS) (Schuckit, 1980) consists of 13 descriptors of alcohol's effects, including uncomfortable, high, clumsy, muddled or confused, slurred speech, dizzy, nauseated, drunk or intoxicated, sleepy, distorted sense of time, effects of alcohol or drug, difficulty concentrating, and feelings of floating. Subjects were instructed to respond to how they felt "right now" using a horizontal line anchored by "not at all" on the left and "extremely" on the right. Based on previous studies that have used this scale to study genetic influences on subjective responses to alcohol (Hu et al., 2005; Ray and Hutchison, 2004; Schuckit et al., 1999), we analyzed *GABRA2* genotype and haplotype effects on "High" and "Drunk or Intoxicated" effects.

Alcohol Levels Determination—In most subjects, BAC was assayed from plasma using an Alcohol analyzer (Analox Instruments, Hammersmith, London). Coefficient of variance for precision is 1–2% and sensitivity is 0.1 mg/dL. In 9 participants in whom plasma was not obtained BAC was estimated from breath using an Alco-Sensor IV Instrument (Intoximeters Inc., St Louis, MO). Correlation of blood and breath alcohol levels was 0.959 ($p < 0.0001$) in 28 participants in which both measures were obtained.

Genotyping Procedures

SNP markers—Genotyping was conducted across 140.2 kb in the chromosome 4 region that contains *GABRA2*, and extending 63.1 kb 3' towards the region where *GABRG1* is located and 8 kb 5'. The intergenic region 3' *GABRA2*–5' *GABRG1* was included based on previous literature showing association of SNPs in this region with AD (Covault et al., 2004; Covault et al., 2007). Twenty-six SNPs were selected from those already characterized in the literature as associated with AD and those identified in the region by the International HapMap Project (<http://www.hapmap.org>), which includes SNPs that have also been registered in The National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nih.gov>). Individual SNPs had to show a minor allele frequency > 0.05 . Linkage disequilibrium (LD) between neighboring SNPs was taken into account by selecting SNPs that pair wise showed an r^2 of < 0.8 . Table 1 lists the characteristics of the SNPs examined in the current study.

Genomic DNA—Genomic DNA was extracted from leukocytes using the Gentra Puregene kit (Quiagen, Minneapolis, MN).

Polymerase Chain Reaction (PCR) amplification of SNPs—Genotyping was performed by the 50-nuclease method using fluorogenic allele-specific probes. Oligonucleotide primer and probe sets were selected from the human TaqMan® Pre-Designed SNP Genotyping Assays or designed using Applied Biosystems' Custom TaqMan® SNP Genotyping Assay service (ABI, Foster City, CA). PCR amplification reactions for Pre-Designed SNPs were performed in 5 μ l volume containing 0.125 μ l 40X assay mix, 2.5 μ l Master Mix, and 7.5 ng genomic DNA. PCR amplification reactions for Custom SNPs were performed in 5 μ l volume containing 0.25 μ l 20X assay mix or 0.125 μ l 40X assay mix, 2.5 μ l Master Mix, and 7.5 ng genomic DNA. Reactions were incubated at 50°C for 2 min and at 95°C for 10 min, and amplified for 40 cycles at 92°C for 15 s and 60°C for 1 min. Allele-specific signals were distinguished by measuring endpoint 6-FAM or VIC fluorescence intensities using an ABI 7900HT Sequence Detection System and genotypes were generated using SDS v2.1 software (Applied Biosystems). As a quality control, 20% of genotypes were repeated. PCR amplification failed or provided ambiguous genotype results in 2.8% of SNP Markers.

Genotype distributions were in Hardy-Weinberg equilibrium (HWE) for all SNPs genotyped (range of p -values = 0.05–1.0).

Genotyping for population markers—To estimate genetic ancestry proportions for each subject, DNA samples were genotyped using a panel of 23 markers with high efficiency at clustering individuals into population subgroups (Smith et al., 2001; Yang et al., 2005). Short tandem repeat markers D1S252, D2S319, D12S352, D17S799, D8S272, D1S196, D7S640, D8S1827, D7S657, D22S274, D5S407, D2S162, D10S197, D11S935, D9S175, and D5S410 were selected from Applied Biosystems (AB, Foster City, CA) Linkage Mapping Set v2.5. Markers D7S2469, D16S3017, D10S1786, D15S1002, D6S1610, and D1S2628 were synthesized by Applied Biosystems with fluorescent dye PET® to allow genotyping in the same lane with the other markers. Samples were PCR amplified in 10µl using 30 ng DNA, 0.5U Taq Gold polymerase (Applied Biosystems), 0.15µM primers, 0.6mM dNTPs (CLP, San Diego, CA), 2.5mM MgCl₂ and 1X buffer supplied by the manufacturer. Reactions were amplified in a Veriti thermocycler (Applied Biosystems, Foster City, CA) at 94°C for 12 minutes followed by 40 cycles of 94°C for 30 sec., 55°C for 30 sec., 72°C for 30 sec., with a final extension at 72°C for 10 minutes. PCR products for the individual markers were pooled before electrophoresis on a 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Data were collected and analyzed with GeneMapper software (Applied Biosystems, Foster City, CA) that calculates fragment length in reference to an internal lane standard Genescan-500 labeled with LIZ. All genotypes were manually checked. Duffy Antigen SNP rs2814778 was genotyped using TaqMan® Pre-Designed SNP Genotyping Assay (Applied Biosystems, Foster City, CA). Samples were PCR amplified in 5µl reaction volume containing 0.125µl 40X assay mix, 2.5µl Master Mix and 8ng DNA. PCR and end point detection of fluorescence were carried out in an ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Fluorescence data were analyzed with SDS v2.1 software. As a quality control, 20% of genotypes were repeated. PCR amplification failed or provided ambiguous genotype results in 4% of population markers and 2% of Duffy Antigen SNP.

Statistical Analysis

Summary values for demographic characteristics are expressed as means (SD) for continuous variables and frequencies (%) for categorical ones. The major outcomes of interest were subjective measurements described above. The correlations among these subjective measures were low and in some cases moderate in magnitude, therefore suggesting that analyzing them separately would yield distinct information.

Population substructure—The population analysis software Structure 2.3.3 (developed by J. Pritchard from University of Chicago) was utilized to check for population genetic substructure in our sample using the 23 markers genotyped as described above. The first two ancestry proportions were adjusted for in the association analysis.

We conducted two different sets of analyses to assess the effect of genotype and haplotypes on the subjective responses to alcohol. Change from baseline in the subjective outcome measures was the dependent variable in the longitudinal analysis. Linear mixed effects models were used to take into account correlation due to multiple measurements per subject. The change scores were normally-distributed. Given that we have a placebo drink during the session at time 0 prior to the three alcohol drinks, this enables within-subject comparison with the placebo condition. In our analysis, we model unstructured time trend from the beginning to the end of the session, in which placebo drink is the reference time point and responses following alcohol drinks subsequent to the placebo drink are all contrasted with this reference. Thus, comparison of the genotype groups takes into account the treatment difference across time.

In addition, we evaluated if the placebo drink was believable for participants. We observed that the “drunk or intoxicated” SHAS subjective response scores were significantly different following placebo drink administration compared to baseline subjective response scores ($p=0.0015$), whereas there was no difference in BAC levels between baseline and following placebo drink administration, with BAC being zero at both time points (Figure 2). Also, we observed significant differences between subjective response scores following placebo drink and all time points following alcohol administration ($p<0.0001$); as expected, the drunk or intoxicated scores increased with the cumulative alcohol drinks until participants reached peak BAC, and decreased following the alcohol drinks as BAC was declining.

A. Individual SNP analysis—The linear mixed effects model included a contrast for genotype differences as a major predictor, while controlling for time, BAC (time-dependent), genetic ancestry proportion as well as demographic characteristics including age, gender and drinking behavior variables that were significant for the outcome of interest. No significant interaction effects between time and genotypes were detected; therefore, the results reported were obtained from models without the interaction term.

Determining significance level for individual SNP analysis—The conventional alpha or Type I error level to declare a finding significant ($p<0.05$) was corrected for multiple tests. Because the analyzed SNPs are in LD with each other, the tests are not independent. Thus, the method proposed by Nyholt (Nyholt, 2004) was used to evaluate the correlation between SNPs to determine the effective number of independent tests in the analysis. To do this, we used Haploview to calculate the number of haplotype blocks included in the candidate gene using the procedures described below. Tagging SNPs that did not fall within a defined block were counted as an independent test. We estimated a total of 8 effective independent tests. Therefore, using a conservative approach, we applied a Bonferroni correction for Type I error for 8 tests ($\alpha = 0.05/8=0.0062$). The tests were two sided with a 0.0062 significance level.

B. Haplotype analysis—Given that our sample was predominantly Caucasian (see Table 2), the haplotype analysis was limited to the 52 individuals who self-reported their race as Caucasian and were also determined to be so based on their genetic ancestry proportion. The linear mixed effects model included a contrast for haplotype differences as a major predictor, while controlling for time, BAC, as well as demographic characteristics including age, gender and drinking behavior variables that were significant for the outcome of interest.

B.1. Reconstruction of haplotypes in the study cohort (Caucasian population)

—The program Haploview (v4.2) was used to delineate haplotype blocks (Barrett et al., 2005). Haploview calculates linkage disequilibrium (D' or r^2) between each pair of SNPs. We used the method of confidence intervals to assign haplotype blocks (Gabriel et al., 2002). For SNPs within the same haplotypic block, haplotypes were reconstructed using the program PHASE2 (Stephens et al., 2001), which employs an iterative stochastic-sampling strategy and the pseudo-Gibbs sampler for assignment of haplotype phases. Based on the haplotype frequency estimation, we assumed a dominant genetic model in Haplotype association analysis. The tests were two sided with a 0.05 significance level.

All association analyses were performed using the SAS software, version 9.2 (SAS Institute Inc, Cary, NC).

Results

1. Demographics

Demographic characteristics for the participants who underwent the alcohol challenge are presented in Table 2. Subjects were healthy, with a mean age of 24 years, an average of 3 years of college, and were predominantly Caucasian. The subjects drank an average of 1.5 days per week, averaging 3 drinks per episode. There were three subjects who were smokers of less than 3 cigarettes per day. Demographic characteristics that predicted the subjective outcome measures being analyzed were adjusted for in our statistical models.

2. Blood alcohol level

As shown in Figure 2, a range of BAC levels obtained over time captured both the ascending and descending limb of the alcohol dose effect curve within the session. In addition, the peak blood alcohol level (mean 100.4 mg/dL, SE =2.5) indicated the target dose of 100 mg/dL was obtained using the cumulative oral alcohol dosing procedure. There were no significant genotype group differences in BAC levels.

3. Individual SNPs effects on Subjective Responses to Alcohol

3. A. Visual Analog Drug Effect Questionnaire (VAS)—During the alcohol challenge session, subjects with one or two copies of the minor alleles for the following SNPs reported on average significantly *lower* “Negative” effects (i.e., the sum of “Bad, Dislike and Worst” effects scores) than those homozygous for the common allele after adjusting for time, BAC, genetic ancestry proportion and number of binge drinking episodes (Table 3). **rs279858:** Negative effects score was lower in carriers of the minor allele, C, than in subjects homozygous for the common allele ($F(1, 62) = 8.10, p=0.0060$) (Figure 3.A.). **rs 279844:** Negative effects score was lower in carriers of the minor allele, T, than in subjects homozygous for the common allele ($F(1, 62) = 9.22, p=0.0035$). **rs 279845:** Negative effects score was lower in carriers of the minor allele, A, than in subjects homozygous for the common allele ($F(1, 61) = 8.70, p=0.0045$). **rs279826:** Negative effects score was lower in carriers of the minor allele, G, than in subjects homozygous for the common allele ($F(1, 61) = 8.80, p=0.0043$). **rs279828:** Negative effects score was lower in carriers of the minor allele, C, than in subjects homozygous for the common allele ($F(1, 63) = 9.07, p=0.0037$). **rs 279836:** Negative effects score was lower in carriers of the minor allele, A, than in subjects homozygous for the common allele ($F(1, 62) = 8.08, p=0.0061$) (Figure 3.B.). When the SNPs described above were analyzed in the Caucasian subsample only, the effects were similar (Table 3).

We did not observe significant association of individual SNPs with alcohol’s positive and stimulant effects, SHAS High and Intoxicated effects or alcohol’s sedative effects.

4. Haplotype block structure in the Caucasian cohort

Two main haplotype blocks were observed (Figure 4). The first haplotype block includes SNPs extending from the intergenic region 5′ *GABRG1-3′ GABRA2* to intron 3 of *GABRA2*. The second haplotype block includes SNPs located in intron 3 of *GABRA2*. Table 4 describes the haplotype blocks and frequencies in our population.

5. Haplotype effects on Subjective Responses to Alcohol

5. A. Block 1: intergenic region 5′ *GABRG1-3′ GABRA2* and *GABRA2* region

5. A.1. Visual Analog Drug Effect Questionnaire (VAS): Carriers of one or two copies of block 1, haplotype 1, GTGGTACCTCTTAGCAGC, had significantly lower mean alcohol “Negative” effects scores (14.18, SE = 3.93) than non-carriers [mean = 23.17 (SE = 3.26),

($F(1, 49) = 4.07, p=0.0492$) (Figure 5). Similarly, carriers of one or two copies of block 1, haplotype 4, ACGATGTTGTTATAATAT, had significantly lower mean alcohol “Negative” effects scores (17.19, SE = 3.84) than non-carriers [mean = 20.86 (SE = 3.30)], after adjusting for time, BAC, and number of binge drinking episodes ($F(1, 49) = 6.36, p=0.0150$) (Table 5).

5. A.2. Biphasic Alcohol Effects Scale (BAES): Carriers of one or two copies of block 1, haplotype 3, ACTATGTTGTTATAATAT, had significantly higher mean alcohol sedative effects scores (5.32, SE = 0.43) than non-carriers [mean = 2.69 (SE = 0.34), after adjusting for time, BAC, and age ($F(1, 49) = 4.94, p=0.0309$)]. In contrast, carriers of one or two copies of block 1, haplotype 4, ACGATGTTGTTATAATAT, had significantly lower mean alcohol sedative effects scores (2.76, SE = 0.40) than non-carriers [mean = 4.84 (SE = 0.36)], after adjusting for time, BAC, and age ($F(1, 49) = 7.62, p=0.0081$) (Table 5).

5. A.3. Subjective High Assessment Scale (SHAS): Carriers of one or two copies of block 1, haplotype 4, ACGATGTTGTTATAATAT, had significantly lower mean alcohol “High” effects (5.77, SE = 1.31) than non-carriers [mean = 16.46 (SE = 1.49), after adjusting for time and BAC ($F(1, 50) = 4.98, p=0.0302$) (Table 5).

5. B. Block 2: *GABRA2* region

5. A.1. Visual Analog Drug Effect Questionnaire (VAS): Carriers of one or two copies of block 2, haplotype 1, GT, had significantly higher mean alcohol “Positive” effects scores (i.e., the sum of “Good, Like and Best” effect scores) of 41.54 (SE = 3.47) than non-carriers, who had mean alcohol “Positive” effects score of -4.46 (SE = 8.50), after adjusting for time and BAC ($F(1, 50) = 6.53, p=0.0137$) (Table 5).

Discussion

This is the first study reporting the association of SNPs and haplotypes within the *GABRA2* region with subjective responses to alcohol using an oral alcohol challenge. The most consistent finding in this study was that carriers of the minor alleles for SNPs rs279858, rs279844, rs279845, rs279826, rs279828 and rs279836 reported lower mean “Negative” alcohol effects scores than individuals homozygous for the common allele. Furthermore, haplotype effects of block 1, which contains the aforementioned SNPs, showed concordant results. The importance of these observations is that the minor allele for several of the SNPs in which we observed lower “Negative” effects of alcohol have been associated with AD in multiple studies across different samples. This supports evidence that individual differences in subjective responses to alcohol as measured by a laboratory alcohol challenge procedure are related to the risk of developing AUDs (Morean and Corbin, 2010; Quinn and Fromme, 2011). Our findings provide further evidence that genetic variations within the *GABRA2* region moderate the subjective responses to alcohol and, thus, may enhance vulnerability to AUDs.

Specifically, in our current study, we observed that carriers of the minor alleles, C for SNP rs279858 and T for SNP rs279844, which have been associated with AD in numerous studies and diverse populations (Covault et al., 2004; Covault et al., 2008; Enoch et al., 2009; Fehr et al., 2006; Lappalainen et al., 2005) had lower “Negative” effects of alcohol. Of note, when examining SNP rs279858, some authors report the minor allele as G, which is comparable to our results with the C allele given that genotyping was performed in the forward strand in our study. Our findings are consistent with the hypothesis that risk of AD in carriers of these minor alleles may, in part, be mediated by the decreased subjective negative responses to alcohol. These findings are in agreement with previous studies

showing that a low level of overall response to alcohol appears to represent an important component of inherited risk for AUDs, supporting the low level of response model (Heath et al., 1999; Schuckit and Smith, 1996, 2000; Trim et al., 2009). Adding further evidence, another study which examined the influence of a single SNP, *rs279858*, on subjective effects of alcohol (Pierucci-Lagha et al., 2005) described that participants homozygotes for the more common allele (comparable to our results with the T allele) reported greater stimulation following alcohol administration than the G-allele carriers (AD associated allele comparable to our results with the C allele) as measured by the BAES and Central Stimulation subscale of the Alcohol Sensation Scale. In this study, the authors interpreted that the decreased subjective response to alcohol of individuals carrying the minor allele, G, may in part mediate the increased risk for AD observed in population studies.

Our haplotypic analysis indicated that carriers of block 1, haplotypes 1 and 4 had lower alcohol “Negative” effects than non-carriers. As expected, carriers of block 1, haplotype 1, a predominantly minor allele haplotype, had concordant results with the analysis of SNPs in this block. Interestingly, carriers of haplotype 4, although a predominantly major allele haplotype, also contained the minor alleles for various SNPs in the haplotype; therefore, these findings are also concordant with the individual SNP analyses.

Because of their ability to use the information inherent in LD, testing associations with haplotypes may be more powerful than with individual SNPs (Chapman and Wijsman, 1998; Chiano and Clayton, 1998; Johnson et al., 2001). We observed associations of block 1, haplotype 4 with subjective “High” effects of alcohol using an uncorrected p-value, and of block 2, haplotype 1, with “Positive” effects of alcohol. However, individual analysis of SNPs that compose these haplotypes did not reveal significant associations with positive and stimulant effects of alcohol. More studies of the association of haplotypes in this region to AD are needed to validate our findings.

One of the strengths of our study is that we used an oral alcohol challenge, and achieved a target peak BAC of 100.4 mg/dL 25 minutes following the cumulative alcohol doses, which allowed us to observe subjective responses on both the ascending and descending limbs of the BAC. In addition, given that subjective responses to alcohol have been found to differ by drinking phenotype (i.e., heavy alcohol consumption or persons at increased risk for AD) (Morean and Corbin, 2010; Quinn and Fromme, 2011), we took into account recent drinking behavior in our analysis and adjusted for these variables when they had a significant effect on the subjective response being evaluated. Also, we observed not only SNP, but also haplotype effects on subjective responses to alcohol measured throughout the whole session. Other strengths of the study include the selection of SNPs spanning the *GABRA2* region including several SNPs that have been consistently associated with AD (Covault et al., 2004; Covault et al., 2008; Enoch et al., 2009; Fehr et al., 2006; Lappalainen et al., 2005), which allowed for a comprehensive examination of *GABRA2* SNPs on subjective responses to alcohol. Furthermore, the number of subjects who participated in our study is relatively large compared to other studies (Pierucci-Lagha et al., 2005), which provided the statistical power necessary to examine a larger group of SNPs. In addition, we were very stringent in setting significance levels. Importantly, we estimated genetic ancestry proportions for each subject to identify population substructure within our sample, and ancestries were adjusted for in the SNP and haplotype association analyses.

The results of our study must be viewed in the context of its limitations. Our findings that individuals carrying the AD-associated alleles for SNPs in the *GABRA2* region experience lower negative responses to alcohol may indicate that these individuals can drink more alcohol without experiencing its negative effects than non-carriers of the minor allele and may therefore be more likely to drink and drink heavily when given access to alcohol. It

could be argued that further studies are needed to determine the effects of *GABRA2* alleles on drinking behavior as it occurs in natural settings or using self-administration paradigms. Another limitation of the study is that the haplotype analyses included only Caucasian subjects; therefore, similar studies are needed in other population groups to ensure that the findings generalize to these groups. Finally, although we observed that SNPs and haplotypes were associated with differences in subjective responses to alcohol, to our knowledge the functional effects of the allelic variants examined remain unknown. Therefore, the SNPs examined may be in LD with variation at other loci within the *GABRA2* gene or genes in close proximity that are responsible for the effects observed in our study. Future research should examine additional polymorphisms within this gene cluster and aim to elucidate the potential mechanisms by which these variants influence the subjective responses to alcohol.

Our findings provide further evidence that genetic variations within the *GABRA2* region moderate the subjective responses to alcohol and thus, may increase vulnerability to AUDs. These findings are important in that they may aid in the identification of individuals at risk for AUDs and those who are the most critical targets for alcohol prevention interventions.

Acknowledgments

Supported by NIH grants AA017466 (M Uhart), and AA020342 and MH076953 (GS Wand).

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Figure 1.

Timeline of events during the Alcohol Challenge Session. At time 0, placebo was administered, using blinding procedures. Three equal doses of alcohol were then administered at 45-min intervals; subjects had 10 minutes to finish each drink. Subjective assessments were obtained at baseline, following each drink, and at 45-minute intervals for the remainder of the session. BAC depicts times at which alcohol concentration was measured.

Figure 2. Blood alcohol concentration achieved over time during the Alcohol Challenge Session. Values reflect mean (SE) time course of blood alcohol level during the alcohol sensitivity session. P denotes time of placebo drink. A denotes times of alcohol drinks.

Figure 3.

A. Visual Analog Drug Effect Questionnaire (VAS) “Negative” effects responses to acute alcohol administration by *GABRA2* SNP rs279858. Values reflect unadjusted means (SE). P denotes time of placebo drink. A denotes times of alcohol drinks. Subjects homozygous (n=14) or heterozygous (n=31) for the minor allele, C, reported on average a lower mean change from baseline “Negative” effects than those homozygous for the common allele (n=23) (p=0.0060).

B. Visual Analog Drug Effect Questionnaire (VAS) “Negative” effects responses to acute alcohol administration by *GABRA2* SNP rs279836. Values reflect unadjusted means (SE). P denotes time of placebo drink. A denotes times of alcohol drinks. Subjects homozygous (n=14) or heterozygous (n=33) for the minor allele, A, reported on average a lower mean change from baseline “Negative” effects than those homozygous for the common allele (n=21) (p=0.0061).

Figure 4.

Pattern of linkage disequilibrium (LD) within the tagging SNPs genotyped across the Chromosome 4 region that contains *GABRA2* in the Caucasian population. The numbers on the top correspond to each SNP's rs number as named in Table 1. Each number in the diamonds represents LD (D') values for the respective SNP pairs. ◆, Absolute LD ($D'=1$); 95, D' : 0.95 between SNPs 3 and 13.

Figure 5.

Visual Analog Drug Effect Questionnaire (VAS) “Negative” effects responses to acute alcohol administration by Haplotype block1, haplotype 1, GTGGTACCTCTTAGCAGC (B1,H1). Values reflect unadjusted means (SE). P denotes time of placebo drink. A denotes times of alcohol drinks. Subjects who were carriers of one or two copies of B1,H1, predominantly a minor allele haplotype (n=24), reported on average significantly lower mean change from baseline alcohol “Negative” effects (which is the sum of “Bad, Dislike and Worst” effects responses) than non-carriers (n=28) (p=0.0492).

Table 1

Characteristics of *GABRA2* SNPs Examined

NCBI SNP (dbSNP build 126)	Genome assembly position (Chromosome 4)	Location in <i>GABRA2</i>	Alleles	MA	MAF
3' <i>GABRA2</i>					
rs 4280776	45883288	63.1 kb 3'	T-C	T	0.331
rs 488447	45887802	58.6 kb 3'	G-A	G	0.457
rs 479277	45893428	53 kb 3'	T-C	C	0.500
rs 478179	45933494	12.9 kb 3'	G-T	T	0.232
rs 567926	45936526	9.9 kb 3'	C-T	C	0.432
<i>GABRA2</i>					
rs 3822051	45951469	Intron	T-C	C	0.179
rs 534459	45951562	Intron	T-C	T	0.493
rs 529826	45966409	Intron	C-T	T	0.478
rs 279871	46000490	Intron	T-C	C	0.433
rs 279869	46002752	Intron	G-T	T	0.493
rs 279858	46009350	Exon (synonymous)	C-T	C	0.434
rs 3775282	46016620	Intron	T-C	C	0.152
rs 279844	46024412	Intron	T-A	T	0.449
rs 279845	46024480	Intron	A-T	A	0.448
rs 279826	46028966	Intron	G-A	G	0.463
rs 279828	46029567	Intron	A-C	C	0.471
rs 279836	46033827	Intron	T-A	A	0.449
rs 279837	46034080	Intron	A-G	G	0.449
rs 1442059	46051709	Intron	C-T	C	0.440
rs 1442060	46060824	Intron	G-A	A	0.492
rs 3849591	46063398	Intron	G-T	T	0.194
rs 13152740	46075978	Intron	G-A	A	0.246
rs 16859354	46080335	Intron	T-G	G	0.311
rs 11503014	46085622	UTR-5	C-G	G	0.311
5' <i>GABRA2</i>					

NCBI SNP (dbSNP build 126)	Genome assembly position (Chromosome 4)	Location in <i>GABRA2</i>	Alleles	MA	MAF
rs 1372472	46090955	4.25 kb 5'	A-T	T	0.339
rs 1025852	46095088	8.38 kb 5'	G-A	A	0.336

MA, minor allele in our study population; MAF, minor allele frequency in our study population.

Table 2

Demographic characteristics

Age, mean (SD), (y)	24.25 (2.63)
Gender, No. (%)	
Male	41 (59.4)
Female	28 (40.6)
Race, No. (%)	
Caucasian, self reported and genetic ancestry proportion	52 (75.4)
Caucasian, self reported	2 (2.9)
African-American, self reported	5 (7.25)
Asian, self reported	7 (10.1)
Other, self reported	3 (4.35)
BMI, mean (SD), (kg/m ²)	24.84 (3.51)
Education, mean (SD), (y)	15.43 (1.13)
Non-Smoker, No. (%)	66 (95.65) ^a
Recent Drinking Behavior, mean(SD)	
Drinking episodes per week	1.52 (0.92)
Drinks per drinking episode	3.10 (1.70)
No. Drinking days in past 90 days	19.42 (11.98)
No. Drinks in past 90 days	57.25 (39.40)
No. Binge drinking episodes in past 90 days	3.41 (4.54)

^aThree smokers of less than 3 cig/day

Relative means (SE) of SNPs effects on subjective measures of alcohol effects. The analysis for all the subjects was adjusted for demographic covariates including genetic ancestry proportion and number of binge drinking episodes. The analysis for Caucasians only was adjusted for number of binge drinking episodes.

Table 3

Subjective Measure	Gene/Region	SNP	Haplotype Block	Minor Allele	MAF	Model*	N0	N1	N2	Overall mean of change from baseline		P-value	Overall mean of change from baseline (Caucasians Only)
										Mean0 (SE)	Mean1 (SE)		Mean0 (SE)
VAS Negative	GABRA2	rs279858	B1	C	0.434	DOM	23	31	14	27.63 (3.74)	16.72 (2.80)	0.0060	26.60 (4.03)
	GABRA2	rs279844	B1	T	0.449	DOM	22	31	15	25.59 (3.77)	17.93 (2.79)	0.0035	26.60 (4.03)
	GABRA2	rs279845	B1	A	0.448	DOM	22	30	15	25.59 (3.77)	19.80 (2.83)	0.0045	26.60 (4.03)
	GABRA2	rs279826	B1	G	0.463	DOM	20	32	15	26.20 (3.92)	19.79 (2.77)	0.0043	24.90 (4.10)
	GABRA2	rs279828	B1	C	0.471	DOM	20	33	16	26.20 (3.92)	18.54 (2.69)	0.0037	24.90 (4.10)
	GABRA2	rs279836	B1	A	0.449	DOM	21	33	14	28.41 (3.89)	16.84 (2.74)	0.0061	24.90 (4.10)

*Model: Dominant (DOM): compare carriers of one or two minor alleles versus non-carriers.

N0: number of participants that are non-carriers of the minor allele; N1: number of participants that carry one minor allele; N2: number of participants that carry two minor alleles

Mean0 (SE) is the overall mean and standard error among non-carriers; Mean1 (SE) is the overall mean and standard error among carriers of one or two minor alleles.

Table 4

Haplotype blocks and frequencies

	Haplotype	Haplotype frequency
Block1 H1	GTGGTACCTCTTAGCAGC	0.241154
Block1 H2	GTGGCACCTCCTAGCAGC	0.132733
Block1 H3	ACTATGTTGTTATAATAT	0.234777
Block1 H4	ACGATGTTGTTATAATAT	0.292999
Block2 H1	GT	0.683293
Block2 H2	AG	0.224732

For each haplotype block, the table shows haplotypes with frequency > 0.1

Block 1 is composed of SNPs rs488447, rs479277, rs478179, rs567926, rs3822051, rs534459, rs529826, rs279871, rs279869, rs279858, rs3775282, rs279844, rs279845, rs279826, rs279828, rs279836, rs279837, rs1442059. Alleles corresponding to each SNP in the block are shown in this order in the table. Block 2 is composed of SNPs rs13152740, rs16859354. Alleles corresponding to each SNP in the block are shown in this order in the table.

Table 5

Relative means (SE) of Haplotype effects on subjective measures of alcohol effects

Subjective Measure	Gene/Region	Haplotype Block	N0	N1	Overall mean of change from baseline		P-value	Demographic covariates adjusted for
					Mean0 (SE)	Mean1 (SE)		
VAS Negative	Intergenic- <i>GABRA2</i>	B1, H1	28	24	23.17 (3.26)	14.18 (3.93)	0.0492	Number of binge drinking episodes
	Intergenic- <i>GABRA2</i>	B1, H4	26	26	20.86 (3.30)	17.19 (3.84)	0.0150	Number of binge drinking episodes
VAS Positive	<i>GABRA2</i>	B2, H1	4	48	-4.46 (8.50)	41.54 (3.47)	0.0137	
Sedation (BAES)	Intergenic- <i>GABRA2</i>	B1, H3	30	22	2.69 (0.34)	5.32 (0.43)	0.0309	Age
	Intergenic- <i>GABRA2</i>	B1, H4	26	26	4.84 (0.36)	2.76 (0.40)	0.0081	Age
SHAS High	Intergenic- <i>GABRA2</i>	B1, H4	26	26	16.46 (1.49)	5.77 (1.31)	0.0302	

N0: number of participants that are non-carriers of the haplotype; N1: number of participants that carry one or two copies of the haplotype.
Mean0 (SE): overall mean and standard error for non-carriers of the haplotype. Mean1 (SE): overall mean and standard error for carriers of 1 or 2 copies of the haplotype.