Published in final edited form as:

Alcohol Clin Exp Res. 2010 December ; 34(12): 2169–2178. doi:10.1111/j.1530-0277.2010.01293.x.

A Polymorphism in *GABRA2* is Associated with the Medial Frontal Response to Alcohol Cues in an fMRI Study

David A. Kareken, Ph.D.^{1,2,3}, Tiebing Liang, Ph.D.⁴, Leah Wetherill, MS⁵, Mario Dziedzic, Ph.D.^{1,2}, Veronique Bragulat, Ph.D.¹, Cari Cox, M.S.¹, Thomas Talavage, Ph.D.^{6,7}, Sean J. O'Connor, M.D.³, and Tatiana Foroud, Ph.D.⁵

¹Department of Neurology, Indiana University School of Medicine, Indianapolis, IN

²Department of Radiology, Indiana University School of Medicine, Indianapolis, IN

³Department of Psychiatry, Indiana University School of Medicine, Indianapolis, IN

⁴Department of Medicine, Indiana University School of Medicine, Indianapolis, IN

⁵Department of Medical & Molecular Genetics Indiana University School of Medicine, Indianapolis, IN

⁶School of Electrical and Computer Engineering, Purdue University, West Lafayette, IN

⁷Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN

Abstract

Background—Significant evidence has accumulated to suggest an association between single nucleotide (SNP) polymorphisms in the *GABRA2* gene and alcoholism. However, research has yet to show an association between these polymorphisms and the human brain's reward system function. In this study, we stratified subjects who had participated in an fMRI study of alcohol cue responses according to their genotype at a SNP in *GABRA2* (rs279871) shown to be associated with alcohol dependence (Edenberg et al., 2004).

Methods—Genotyping showed 13 subjects to be homozygous for the high-risk allele (AA), and 23 subjects to be heterozygous (AG). In fMRI, subjects were exposed to the aromas of their preferred alcoholic drink odors (AO), as well as to appetitive control odors (ApCO) under both alcohol intoxication and placebo control conditions.

Results—Homozygous AA subjects had a larger [AO > ApCO] response than did AG subjects in medial frontal cortical areas thought to code reward value. However, AG subjects had a larger [AO > ApCO] effect in the ventral tegmental area. Alcohol intoxication did not alter these group differences.

Conclusions—These are the first data to suggest that *GABRA2* genotype could affect the brain's reward responses to cues associated with alcohol.

Introduction

One of alcohol's several central nervous system mechanisms is its affinity for γ -aminobutyric acid (GABA) receptors of the A type (GABA-A). These ligand-gated receptors are involved in most inhibitory neural transmission, and commonly exist in pentameric configurations of two α , two β , and one γ subunits. $\alpha 2$ subunits, which populate limbic and reward-related brain areas, such as the ventral tegmental area (VTA) and nucleus accumbens (Pirker et al., 2000), mediate the anxiolytic effects of benzodiazepines (with which alcohol is cross tolerant), but not its amnestic or sedative effects (Löw et al., 2000). Data specific to alcohol are somewhat more ambiguous (Täuber et al., 2003), but knockout mice lacking the $\alpha 2$ subunit may be less sensitive to alcohol's hypnotic effects (Boehm II et al., 2004).

Against this background, Edenberg (2004) genotyped SNPs across the GABA cluster on chromosome 4p and reported evidence of an association between alcoholism and single nucleotide polymorphisms (SNP) in the *GABRA2* gene, which codes the GABA-A receptor's $\alpha 2$ subunit. This same report also noted an association between β -EEG oscillations and *GABRA2* variation. The association of SNPs in *GABRA2* and alcohol dependence has been replicated in several independent reports (Covault et al., 2004; Lappalainen et al., 2005; Soyka et al., 2008). Subsequent analyses using the same dataset as Edenberg et al (2004) have refined the association: Agrawal et al. (2006) reported that the *GABRA2* association was limited to alcoholism with co-morbid drug dependence, while in analyses of adolescents Dick et al. (2006) found that SNPs in *GABRA2* were associated with conduct disorder but not alcoholism. Other studies have found no association between alcoholism and the *GABRA2* gene (Enoch et al., 2009; Enoch et al., 2010; Onori et al., in press), or have found the *GABRA2* association with alcoholism to be mediated by anxiety (Enoch et al., 2006). Nevertheless, others report a relationship between *GABRA2* and reduced pleasurable effects from alcohol (Pierucci-Lagha et al., 2005), early onset and familial alcoholism (Fehr et al., 2006), drinking frequency in alcoholics in treatment (Bauer et al., 2007), and alcoholic withdrawal signs (Soyka et al., 2008). Although no apparent coding differences have been found in *GABRA2* that could account for the reported association (Edenberg et al., 2004), Hurley et al (2009) mimicked gene expression by altering the relative concentrations of GABA-A subunits— a manipulation that changed GABA current amplitudes both in the absence of alcohol as well as during alcohol exposure. Thus, given associations between *GABRA2* gene variation and alcoholism, the response to alcohol, related behavioral characteristics, and a particular β -EEG endophenotype, this gene seems a promising candidate to pursue in human neuroimaging studies.

After the repeated pairing of alcohol's pharmacologic actions with its other sensory properties, learned associations can develop between intoxication and alcohol's taste, smell, and sight. Such Pavlovian “conditioned stimuli” are thought to be important in the maintenance of drinking, craving, and relapse (Carter & Tiffany, 1999; Cooney et al., 1997; Litt et al., 2000; Cox et al., 2002). Since particular cerebral vulnerabilities that affect the response to alcohol may be inherited (Schuckit, 2009), it is also plausible that the brain's response to alcohol's conditioned stimuli may be similarly influenced by genetic history (Tapert et al., 2003; Katner et al., 1996). Using functional magnetic resonance imaging

(fMRI) in non-dependent subjects matched for heavy “at-risk” drinking, we recently showed that a family history of alcoholism significantly affects medial frontal reward region responses to the odors of subjects’ preferred alcoholic drinks (Kareken et al., 2010). In particular, heavy drinking subjects who were family history positive for alcoholism had a greater response to alcoholic drink odors (AO, as compared to odors of grape and chocolate) than did family history negative subjects. This differential response occurred in medial frontal areas shown by others (Hare et al., 2009) to code for perceived reward value. The group difference in response also interacted with alcohol intoxication, which potentiated the AO response in family history negative subjects, and reduced it in family history positive subjects. Thus, similar to animal models showing that selective breeding for ethanol preference leads to significant differences in reward system function and constitution (Murphy et al., 2002), genetic factors may affect the human reward system response to alcohol and alcohol-related stimuli.

In the study presented here, we stratified subjects based on their genotype at SNP rs279871 in *GABRA2*, which was previously shown to be associated with alcohol dependence (Edenberg et al., 2004). Given data pointing to a dose dependent allelic effect with SNPs in *GABRA2* (Covault et al., 2004; Fehr et al., 2006), we hypothesized that drinkers homozygous for the high-risk allele would show a greater response to AO (as compared to a baseline of appetitive control odors; ApCO) than drinkers carrying only one copy of the risk allele. We focused our hypotheses on medial prefrontal cortex, where we had noted an association between family history and blood oxygenation level dependent (BOLD) contrast sensitive responses to these stimuli. We also examined other regions, *post-hoc*, including the VTA, ventral striatum, and caudate heads. In addition, we investigated how alcohol intoxication from intravenous alcohol administered during imaging interacted with *GABRA2* genotype.

Subjects

Subjects were recruited and assessed using the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA; Bucholz et al., 1994), the Timeline Followback interview for habitual drinking (TFLB; Sobell et al., 1986), and the Alcohol Use Disorders Identification Test (AUDIT; Saunders et al., 1993). The sample reported here includes subjects studied in Kareken, et al. (2010), except for three subjects whose blood samples were unavailable for genotyping. Also included were 14 social drinkers and four subjects whose SSAGA responses satisfied DSM-IV criteria for alcohol dependence (drinks/week, range 14-32 for these latter four subjects). None of the subjects in this study were undergoing treatment for substance abuse/dependence, and none expressed any interest in such treatment at the time of study.

Based on the extensive linkage disequilibrium (LD) in *GABRA2*, results from previous studies were reviewed and a single SNP, rs279871, was selected for genotyping (Edenberg et al., 2004). Blood samples were analyzed at the Indiana University Alcohol Research Center. DNA was isolated using the “HotSHOT” method (Truett et al., 2000) where TaqMan probes are used for allelic discrimination (Applied BioSystems, Foster City, CA).

After genotyping, there were 13 AA, 23 AG, and 4 GG genotypes available in a sample that was 91% Caucasian. This SNP was in Hardy Weinberg equilibrium in the combined sample ($p=0.18$) as well as in the Caucasian subset ($p=0.55$). Results with SNPs in high LD with rs279871 ($r^2 > 0.70$) suggest that there may be an allelic dosage effect, with those individuals homozygous for the high risk allele having the greatest risk for alcohol dependence (Covault et al., 2004; Fehr et al., 2006). For this reason, all statistical analyses compared the subjects homozygous for the high risk allele at rs279871 (A, allele frequency = 0.61) to those heterozygous for this allele. Rather than combining the small group of four subjects who did not carry a high risk allele with the rs279871 heterozygotes, these subjects were removed from the primary analyses, but included with the AG heterozygotes in secondary analyses.

When stratified by rs279871 genotype, there were no significant differences between subjects with AA and AG genotypes in age, gender, education, family history status, family history density, AUDIT scores, smoking, or drinking patterns (Table 1). None of the subjects had evidence of Axis-I psychiatric or neurological disorders of the brain or failed olfactory screening. Although all subjects denied using illicit drugs, one AG subject tested positive for cannabinoids on the placebo day. One AG subject tested positive for amphetamines on both placebo and alcohol days, and one AA subject tested positive for amphetamines only on the alcohol day. This latter subject was excluded from analyses of the alcohol session data because of obvious acute intoxication and difficulty detecting odors on that day. No subject otherwise exhibited obvious cognitive or behavioral abnormalities in either session. An additional AA subject's fMRI data could not be used for the alcohol session because of excessive head motion during imaging. This resulted in 13 AA and 23 AG subjects available for image analysis in placebo scans, and 11 AA and 23 AG functional imaging datasets when scanning under alcohol infusion.

All subjects were right handed. All voluntarily signed informed consent statements approved by the Indiana University School of Medicine IRB.

Procedure

Procedures for subject stimulation and imaging were as described in Kareken, et al. (2010). In brief, two fMRI sessions were conducted on separate days at a standardized time (~2:00 pm), and employed the odors of each subjects' preferred alcoholic drinks, in addition to two sets of control odorants. Subjects were asked to refrain from drinking alcohol for 3 days prior to study. Prior to imaging, subjects were provided a light, standardized lunch in the Indiana University General Clinical Research Center. For each session, subjects underwent intravenous infusion of either alcohol or placebo (saline) in a randomized order. To minimize expectations, subjects were told that they could receive alcohol or placebo at any session (one session did not predict the other).

Olfactory stimuli—Odorants were delivered with an air-dilution olfactometer as described elsewhere (Kareken et al., 2004; Bragulat et al., 2008; Kareken et al., 2010). The odorant classes were: **1) Alcohol odors** (AO, each subject's two most frequently consumed alcoholic drinks, except for vodka, which has little detectable odor), **2) Appetitive control odors**

(ApCO; chocolate and grape juice; McCormick & Company, Inc., Hunt Valley, MD), and **3) Non-appetitive odors** (NApO; International Flavors & Fragrances, Union Beach, NJ), representing stimuli that are not evocative of ingestive thoughts or behavior. As some found certain NApO unpleasant, subjects could choose two of four from: grass, leather, lilac and Douglas fir. AO were actual alcoholic drinks rendered volatile by passing an airstream through the drink's liquid in the olfactometer's odorant vial. NApO and ApCO were chosen according to prior data that showed them to be approximately equal in intensity, pleasantness, and representativeness (Bragulat et al., 2008).

Stimulus training and craving—Before entering the scanner room, subjects were told that they would be “familiarized with the odorants” by smelling each (grouped by AO, NApO, ApCO) through the olfactometer while simultaneously seeing representative pictures on a computer monitor. Just prior to combined odor/picture cue-exposure (baseline), and again after each of the three stimulus classes, subjects rated desire to drink by responding to items 11 (“I have an urge to drink now”), 18 (“Nothing would be better than drinking right now”), 21 (“I want to use alcohol right now.”), and 32 (“It would be great to use alcohol now”) from the Alcohol Craving Questionnaire (ACQ; Singleton et al., 2000) on a visual analog scale (VAS; 1= strongly disagree, 7= strongly agree). Subjects rated mood (“Right now, I feel angry, grouchy, annoyed, bad-tempered,” “Right now, I feel happy, energetic, full of pep, cheerful, vigorous”) on the same VAS.

Activation paradigm—Three functional imaging scans of olfactory stimulation per subject-session were performed (24 odor events in each of the three stimulus classes of AO, ApCO and NApO plus 42 odorless control events; see online supplementary figure). No pictures were presented during imaging, and olfactory stimulation was done with the eyes closed. Subjects reported the presence (button 1) or absence (button 2) of an odorant on an MRI-compatible response box (Current Designs, Inc., Philadelphia, PA), but were not asked to identify the odorants.

Odor ratings—After the completion of each imaging session, subjects were re-exposed to the odors to rate odorant intensity, pleasantness, and representativeness (how well the odor represented its intended source) on a 9-point VAS.

Alcohol Administration—Subjects were intravenously infused with either alcohol (6% vol/vol in half-normal saline) or saline (placebo) in a randomized order as previously described (Kareken et al., 2010; Bragulat et al., 2008). Infusion pump rates were computer-controlled, with infusion profiles customized for each individual to achieve the same time course of breath alcohol concentration (BrAC): A linear rise to 50 mg% in 10 min, followed by constant exposure at 50 mg% throughout the approximate 45 minute functional imaging protocol. The placebo infusion employed the same pump-rate profile to be used (or as previously used) in the individual's alcohol session, but infused only saline (see O'Connor et al., 1998; Plawecki et al., 2007; Ramchandani et al., 1999). BrAC was measured immediately after the subject's arrival at Indiana University's General Clinical Research Center, and outside the scanner room immediately after imaging while the IV pumps continued to run.

Subjects rated subjective responses to the infusions on the “high” (operationally to subjects as, “up-stimulated, feeling good”) and “intoxicated” (“drunk, tipsy, inebriated”) items of a modified (oral) Subjective High Assessment Scale (SHAS; Schuckit et al., 2000). Before starting the infusion pump, subjects used a uniform baseline of zero, with ratings subsequently varying from the 0 baseline to a maximum of 100 (the most “high” or “intoxicated” ever). Ratings were obtained at baseline, after reaching the calculated BrAC target before functional imaging, between each functional scan, and once just after the last functional imaging scan.

Image acquisition and analysis—Three whole-brain BOLD contrast sensitive functional imaging scans were conducted on a Siemens 3T Magnetom Trio scanner (Erlangen, Germany). A whole-brain high resolution anatomical image volume (1.0×1.0×1.2 mm voxels) was first collected using a 3D magnetization prepared rapid gradient echo (MPRAGE) sequence for anatomic registration of the functional images. For each functional scan, BOLD volumes of 37 slices covering a 111mm superior-inferior extent of the brain were acquired over a 402s period, using a gradient echo echo-planar imaging pulse sequence that incorporated a 3D prospective acquisition correction to minimize effects of the head motion. The imaging parameters were: 96 × 96 acquisition matrix, 2.5×2.5×3.0 mm voxels; for 17 subjects: 134 measurements, 3000/40 ms repetition/echo time, 90° flip angle, no acceleration, 2.5 mm slice thickness with 0.5 mm interslice gap; For 19 subjects: 174 measurements, 2250/30 ms repetition/echo time, 78° flip angle, GRAPPA acceleration factor of 2, 3.0 mm slice thickness with no inter-slice gap. These minor acquisition differences were necessary given an upgrade to the Trio. Whole-brain voxel-wise testing of the two acquisitions showed no significant differences in BOLD activation to olfactory stimulation using the appetitively neutral NApO ($p < 0.05$, false discovery rate corrected).

Data were analyzed using SPM5 (Wellcome Department of Imaging Neuroscience, University College, London). Functional volumes were corrected for differences in slice acquisition timing and rigid-body realigned to the initial volume of the first functional imaging scan to account for residual movement after prospective motion correction (Thesen et al., 2000). Each subject's high resolution anatomic image was co-registered to the reference functional volume, segmented into gray, white and cerebrospinal fluid tissue components. Nonlinear spatial transformation parameters from this segmentation were subsequently applied to transform functional image volumes into the Montreal Neurological Institute (MNI) coordinate space, which were re-sampled to isotropic 2 mm voxels and smoothed by a 6 mm full-width at half-maximum (FWHM) isotropic Gaussian kernel.

Discrete 2 s periods of odorant (or sham) valve events were modeled in a within-subject general linear model, using as basis functions SPM's canonical hemodynamic response function (HRF). Initial testing showed that primary olfactory cortex (piriform) and olfactory association cortex (orbitofrontal) responses to odorants were maximized when the HRF onset was delayed by one second after the sniff instruction, with time and dispersion derivatives of the HRF accounting for slight variations in response onset and duration. Movement parameters from realignment were included as regressors to account for residual movement-induced effects. A high-pass filter with a cut-off of 1/128 Hz was applied to each voxel's time series to remove low frequency noise; auto-regression was not used with the

long inter-stimulus interval (Della-Maggiore et al., 2002). This within-subject model yielded contrast images of activation within an odorant condition (AO, NApO, and ApCO), with each odorant set contrasted against sniffing of odorless control events (i.e., control valve opening without odorant delivery).

Random effects analyses of the contrast HRF-related images in *a priori* regions of interest (ROIs) used a Genotype(2) $\times$ Odor(2) $\times$ Condition(2) linear mixed-effects models in SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). “Genotype” represents homo- or heterozygosity for the *GABRA2* risk allele (AA and AG), “Odor” refers to activation from AO and ApCO (each contrasted against the odorless control events), and “Condition” reflects alcohol and placebo infusion. We concentrated analyses on AO and ApCO, which represent two classes of appetitive stimuli. Given alcohol's vasoactive properties and the BOLD contrast mechanism's reliance on blood flow and volume, NApO were reserved to test whether alcohol altered the sensory response in primary olfactory (piriform) cortex using functionally defined regions of activation under placebo (see Kareken, et al., 2010). Since alcohol can enhance hunger (Caton et al., 2007; Yeomans et al., 1999), the use of stimuli that should not evoke ingestive behavior/cognition (i.e., NApO) helped to avoid potential interactions between alcohol's appetitive qualities and the primary sensory response.

Our primary hypotheses concerned the *a priori* ROIs of medial (mPFC) and ventromedial prefrontal cortex (vmPFC). These regions were sensitive to the [AO > ApCO] effect, with the right mPFC's [AO > ApCO] response discriminating between groups varying in a family history of alcoholism (Kareken, et al., 2010). Left and right medial frontal ROIs were those used in Kareken et al (2010), and have stereotactic boundaries that approximate the medial prefrontal regions to which the VTA and ventral striatum project (Chiba et al., 2001; Haber et al., 2006; Williams & Goldman-Rakic, 1998). These ROIs encompass activation from reward-associated stimuli in multiple studies (Filbey et al., 2008; Hare et al., 2008; Kable & Glimcher, 2007; McClure et al., 2007; Myrick et al., 2008; Schott et al., 2008), including a prior study by our group using a different sample (Bragulat et al., 2008). The ROIs defining the medial prefrontal areas have rostro-caudal extents spanning +56 mm to +36 mm in MNI space. mPFC has a superior extent of +14 mm and an inferior extent of –6 mm, while vmPFC spans –6 mm to –22 mm. Lateral boundaries were defined by conjoining the ROI boxes with the gray matter voxels in a smoothed (6 mm FWHM) gray matter mask.

Although our primary hypotheses involved these medial frontal regions (in which the prior analysis of family history pointed to evidence of a genetic effect), we also examined other regions *post-hoc*, including the VTA, ventral striatum, and caudate heads, using ROIs as described in Kareken et al. (2010). The MarsBar utility (Brett, et al, 2002; <http://marsbar.sourceforge.net/>) was used to extract each subject's mean AO and ApCO contrast value (i.e., the output of the subject-specific fixed effect model, each relative to odorless sniffing) by averaging across all voxels within each of the above ROIs. These average contrast values were then analyzed in the linear mixed-effects models described above.

Results

Odorant characteristics

Analyzed in a linear mixed-effect model (Genotype[2] $\times$ Odor[3] $\times$ Condition[2]), there was a significant main effect for perceived odor intensity ($F = 9.25_{2,34}$, $p < 0.005$), but without other main effects or interactions. Although there were pair-wise differences ($p < 0.05$) between all odors, NApO were perceived as the least intense on the 0 – 9 point VAS (6.8, SD= 1.4), with AO (7.2, SD= 1.1) and ApCO (7.5, SD= 1.0) being closer to one another. There were no group differences (i.e., Genotype $\times$ Odor interactions) in perceived intensity. There were no significant main effects or interactions for pleasantness, but there was a trend ($F = 3.23_{2,32}$, $p < 0.06$) toward a three-way Genotype $\times$ Odor $\times$ Condition interaction, wherein only during placebo did AA subjects perceive AO as more pleasant (7.6, SD= 1.0) than did AG subjects (6.7, SD= 1.5, $p < 0.05$). ApCO were perceived as equally pleasant across genotypes (AA: 7.2, SD= 1.1; AG: 7.6, SD=1.0). During the alcohol session, there were no group differences. A significant main effect ($F = 7.86_{2,35}$, $p < 0.005$) of representativeness (how well subjects thought the aromas reflected their intended sources) indicated that ApCO (7.1, SD= 1.5) were perceived as less representative than AO (8.0, SD= 1.1) or NApO (7.8, SD= 1.0; p 's < 0.005), but again without any differences across genotypes.

Effects of Alcohol

BrAC was uniformly zero before imaging on both session days in all subjects. BrAC measured immediately after subjects left the scanner room during the IV alcohol infusion session was 0.054 for AA genotypes (SD= 0.005) and 0.052 (SD= 0.007) for AG genotypes, a difference that was not significant. During placebo infusion, subjects rated being “high” and “intoxicated” as uniformly low (means < 3) on the 0 – 100 scale of the SHAS. As tested in a Genotype(2) $\times$ Time(4) linear mixed-effects model (using the four time points after the uniform ‘0’ baseline), there was a significant Genotype effect ($F = 5.14_{1,34}$, $p < 0.05$), with AA subjects feeling less high throughout (Figure 1, top). Employing the presence of an alcohol use disorder as a covariate to address the possible effects of tolerance did not change the significance of the genotype differences for perceived “high.” While there was a significant main effect of Time ($F = 9.54_{1,28}$, $p < 0.05$) that reflected progressive increases from the first time point after baseline, the Genotype $\times$ Time interaction was not significant. There was a significant Time main effect for perceived “Intoxication,” ($F = 9.45_{1,28}$, $p < 0.001$), but neither the Genotype main effect nor the Genotype $\times$ Time interaction were significant.

Piriform (primary olfactory cortical) responses were examined in a Genotype(2) $\times$ Condition(2) linear mixed-effects model using NApO, with condition being a repeated measure. As with the smaller sample (Kareken et al., 2010), alcohol infusion did not induce significant changes in either the left or right piriform BOLD response to NApO in this larger group. This suggests that clamped alcohol did not fundamentally change the observed BOLD contrast. There was also no main effect of Genotype in left or right piriform cortex, suggesting no genotype differences in the basic cortical olfactory sensory response.

Craving/Mood

Desire to drink as a function of exposure to combined odors/pictures during the pre-imaging familiarization procedure was assessed with a Genotype(2) $\times$ Stimulus(4) $\times$ Condition(2) linear mixed-effects model. Condition and stimulus were repeated measures, with stimulus comprising pre-stimulus baseline, as well as the three odorant types accompanied by their representative photos. This model showed a significant main effect for Stimulus ($F=11.62_{3,34}$, $p<0.001$; Figure 2), with AO and related images being the only stimulus set that was significantly different than baseline ($p<0.001$). The main effects for Condition and Genotype were insignificant (i.e., there were no differences in craving by genotype), as were the interaction terms.

Stimulus effects on mood as rated on the 7-point VAS were measured in a similar linear mixed-effects model. Positive mood (“happy, energetic, full of pep, cheerful, vigorous”) was rated as 4.1 (SD=1.1) across all subjects, stimuli and conditions, with no significant main effects of Condition or interactions. There was a significant Genotype main effect ($F=3.55_{3,34}$, $p<0.05$) such that AA subjects rated their mood as slightly more positive (4.6, SD=0.9) than AG subjects (3.9, SD= 1.1). A significant Stimulus effect ($p<0.05$) reflected very small mean increments (0.1 to 0.2; p 's <0.05) in positive mood following AO/related image exposure as compared to the remaining conditions, but which did not differ by genotype. Negative mood (“angry, grouchy, annoyed, bad-tempered”) was rated quite low overall (1.3, SD= 0.6), with the only significant model effect being a Stimulus main effect ($F=3.02_{3,29}$, $p<0.05$), which showed a similarly small mean decrement (−0.1 to −0.2; $p<0.05$) in negative mood after AO and related image exposure as compared to baseline and ApCO/related images.

BOLD activation in medial prefrontal cortex

BOLD responses within each *a priori* ROI (left and right mPFC, vmPFC) were tested in a Genotype(2) $\times$ Odor(2) $\times$ Condition(2) linear mixed-effects model in which Genotype represented *GABRA2* SNP genotype (AA, AG), Odor represented AO and ApCO, and Condition represented alcohol or saline (placebo) infusion. All regions showed significant main effects of Odor (left mPFC, $F=12.48_{1,99}$, $p=0.001$; right mPFC, $F=6.48_{1,34}$, $p<0.05$; left vmPFC, $F=17.01_{1,99}$, $p<0.001$; right vmPFC, $F=6.53_{1,99}$, $p<0.05$) reflecting a larger BOLD response to AO than ApCO (Figure 3a). The only region in which there was a significant Condition effect was the right mPFC ($F=6.08_{1,35}$, $p<0.05$), where the odorant response (combined AO and ApCO contrasted against odorless sniffing) was larger during placebo (mean BOLD contrast 0.66, SD=1.74) than during alcohol infusion (0.00, SD=1.42). However, Condition did not interact with Odor class (indicating that alcohol did not influence AO and ApCO differentially) or with group (indicating that the alcohol effect was equal across genotypes on the general odorant response).

The effect of principal interest, the Genotype $\times$ Odor interaction (i.e., effect of genotype as a function of odorant type), was significant for the left mPFC ($F=6.77_{1,99}$, $p<0.05$), the left vmPFC ($F=4.36_{1,99}$, $p<0.05$), and the right vmPFC ($F=7.45_{1,99}$, $p<0.01$), all reflecting greater responses to AO (compared to ApCO) in subjects with the AA genotype compared to those with AG genotypes (Figure 3b); a similar trend effect was apparent in the right

mPFC ($F=4.06_{1,34}$, $p<0.06$). There was, however, no main effect of Genotype to suggest genotypic group differences that were independent of odor type.

Other regions of interest

From analyses exploring the VTA, ventral striatum, and caudate ROIs, the only significant results were a marginal main effect of Odor in the VTA ($F=4.04_{3,35}$, $p<0.06$) and a significant Genotype $\times$ Odor interaction ($F=4.38_{1,34}$, $p<0.05$). In this case, however, the Genotype $\times$ Odor interaction reflected a greater AO response (compared to ApCO) among AG genotypes than in AA genotypes (Figure 4).

Discussion

Alcoholism has long been known as a familial disease (Cotton, 1979), albeit most likely as the result of many different genes interacting with each other and the environment (e.g., Heath et al., 1997). While the effect of any one gene is likely to have a limited contribution, SNPs in *GABRA2* have been repeatedly associated with alcoholism. In the current study, drinkers who were homozygous for the high-risk allele for one polymorphism in *GABRA2* (AA) had a higher medial frontal BOLD response to preferred alcoholic drink aromas than did heterozygous AG subjects. However, individuals with the AG genotype had a stronger AO response in the VTA.

The medial frontal cortical regions where these differential responses to drink cues occurred are part of the mesocorticolimbic reward circuit. In particular, medial frontal cortex receives projections from both the VTA and nucleus accumbens (Chiba et al., 2001; Haber et al., 2006; Williams & Goldman-Rakic, 1998). Functional neuroimaging also suggests that BOLD responses in medial prefrontal cortex reflect the subjective value that subjects assign to food (Hare et al., 2009; Hare et al., 2008) and money (Kable & Glimcher, 2007; McClure et al., 2004). It is also a region that we (Bragulat et al., 2008) and others (Filbey et al., 2008; Myrick et al., 2008) find to respond to alcohol cues. In a subset of the sample reported here (Kareken et al., 2010), the [AO > ApCO] BOLD contrast in these frontal reward areas was greater in heavy drinkers with a family history of alcoholism, as compared to equally heavy drinkers without such a family history.

That AA genotypes had a stronger BOLD response to AO in medial prefrontal cortex, but a weaker AO response in the VTA (both relative to ApCO), could seem somewhat at odds—particularly if one interprets the VTA as encoding appetitive stimuli that are also processed in medial frontal cortex. However, a precise interpretation of BOLD responses in the VTA is more complex in light of recent findings. In particular, VTA dopaminergic and other midbrain neurons respond to rewarding *and* aversive stimuli, and in both excitatory *and* inhibitory manners (Matsumoto & Hikosaka, 2009; Brischoux et al., 2009; Ungless et al., 2004). It is also possible that local inhibitory responses within the VTA could produce a significant BOLD response, even without excitatory responses from dopamine neurons (Düzel et al., 2009), particularly when considering that the BOLD contrast mechanism does not closely track single or multi-unit transient or phasic spiking (Goense & Logothetis, 2008).

Alcohol exposure during brain imaging did not alter the [AO > ApCO] BOLD contrast as a function of genotype, as it did with family history (see Kareken et al., 2010). In particular, AA genotypes responded more strongly than AGs on both days, irrespective of infusion type. There were, however, genotypic effects in the subjective response to alcohol during imaging, with the putatively higher risk AA subjects reporting a significantly lesser feeling of being 'high' (defined to subjects as 'up, stimulated, feeling good') than AG subjects. Although a similar relative relationship was apparent with the subjective response variable "Intoxicated" (defined as 'drunk, inebriated, tipsy'), the difference was not statistically significant. The direction of the effect was similar to another study of *GABRA2* effects by Pierucci-Lagha (2005), in which 7 homozygous low-risk subjects reported greater stimulant and gastrointestinal effects than 20 subjects with either one or two copies of the risk allele (but also see Lind et al., 2008 for a larger, negative study.).

Why and how *GABRA2* might affect the brain's reward system is uncertain. The variation in *GABRA2* that has been associated with alcoholism occurs in non-coding regions of the gene, and presumably affects gene expression. In that respect, differential expression of GABA-A $\alpha 2$ subunits has the potential to affect GABA currents (inhibitory neurotransmission) both alone and in the presence of alcohol (Hurley et al., 2009). After repeated pairing between alcohol's pharmacologic effects and its other closely linked sensory properties, such as smell and taste, it is conceivable that differential GABA signaling could alter reward system responses to these classically conditioned cues. In similar vein of thought, Hemby et al (2006) showed that chronic ethanol exposure in primates altered GABA-A $\alpha 2$ subunit mRNA expression in orbitofrontal cortex, which these authors theorized could play a role in altering the perception of and response to stimulus salience. On the other hand, *GABRA2* variation could affect broader parameters, such as personality and behavioral inhibition (Dick et al., 2006; Lind et al., 2008), particularly if the *GABRA2* association with alcohol disorders is more prominent in polysubstance abuse (Agrawal et al., 2006), mediated by anxiety (Enoch et al., 2006), or associated with a more generalized addiction potential (Philibert et al., 2009).

There are limitations to this study. The *GABRA2* genotype groups are mixed with regard to recent drinking, family history, and race/ethnicity, and complete stratification along these parameters was not possible with the sample size. However, the subject samples with one or two copies of the risk allele did not differ significantly with regard to family history, drinking, and the presence of an alcohol use disorder. We nevertheless conducted several other analyses to further probe the potential influence of these variables. When using the presence of an alcohol use disorder as a covariate (a variable that was highly collinear with drinks/week, and a better model fit), the significance of the Genotype $\times$ Odor interactions was unchanged in the medial frontal ROIs that showed genotypic effects. The same was true when removing the all three subjects who tested positive for any illicit drug use.

Given the sample size, it was not feasible to include the less frequently available GG genotype as a separate group. In that regard it should be noted that there are data linking the G allele of rs279871 to increased risk of alcoholism (e.g., Fehr, et al., 2006). Therefore, secondary analyses were performed, adding these four GG subjects to the AG group. This resulted in the left vmPFC changing from $p < 0.05$ to $p < 0.06$, and the left mPFC and right

vmPFC genotype differences remaining significant. Future research including more GG subjects is needed to better understand the relationship between this genotype and phenotype.

Since there was a marginal ($p < 0.06$) complex three-way Genotype $\times$ Odor $\times$ Condition interaction in which perceived pleasantness of AO differed according to genotype during placebo, we also employed odorant pleasantness as a covariate. The Genotype $\times$ Odor interaction in the left mPFC and the right vmPFC remained significant, but the interaction in the left vmPFC became insignificant. Even so, it may be premature to entirely dismiss such genotype differences in perceived stimulus pleasantness as pertaining to an extraneous psychophysical stimulus feature, since “pleasantness” may encompass appetitive perceptions of the stimulus. The genotype effect in the VTA was less robust to alternative analyses, and became a trend when adding GG subjects or when removing the three subjects testing positive for any illicit drugs (p 's < 0.06). While not affected by including the alcohol use disorder covariate, the genotype effect in the VTA also became insignificant when using perceived odorant pleasantness as a covariate.

In summary, BOLD responses to preferred alcoholic drink odors in medial prefrontal cortical cortex differed along a gradient of “allele dose” in this *GABRA2* polymorphism. Specifically, subjects who were homozygous for the A risk allele had a larger response to AO as compared to ApCO. The medial frontal regions in which this occurred (most robustly left mPFC, right vmPFC) are proximate to the right mPFC region that discriminated between a family history of alcoholism in a subsample of non-dependent heavy drinkers (Kareken et al., 2010). However, a genotype difference occurred in the opposite direction in the VTA, where AO (compared to ApCO) responses were greater in individuals with only one copy of the risk allele. This finding did not remain significant in some analytic scenarios, and the VTA region did not prove sensitive to a family history of alcoholism. These results raise the possibility that polymorphisms in the *GABRA2* gene, or perhaps in adjacent genes in high linkage disequilibrium with *GABRA2* (Enoch et al., 2009), may alter the mesocorticolimbic circuit's sensitivity to reward cues, particularly in medial prefrontal areas shown to encode subjectively perceived reward value (Hare et al., 2008; Hare et al., 2009). To our knowledge, this is the first study to examine the effects of a *GABRA2* polymorphism using functional neuroimaging. However, future research is needed to replicate this finding in larger samples, and in groups that are more homogeneous in drinking, family history, and race.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by R01 AA014605 (DAK), R21 AA018020 (DAK), the Indiana Alcohol Research Center P60 AA007611, and the General Clinical Research Center at Indiana University School of Medicine, M01 RR000750. We gratefully acknowledge the support of Dr. John Nurnberger (Department of Psychiatry), Michele Beal, Victoria Stapleton and Courtney Robbins for imaging (Department of Radiology), Tammy Graves for genotyping, Stephen Warrenburg of International Flavors & Fragrances, and Jaroslaw Harezlak, Ph.D. for statistical consultation.

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Figure 1. Subjective responses to alcohol

Perceived 'High' and 'Intoxication' during alcohol infusion in fMRI using a modified SHAS scale. Baseline= before the infusion pumps are started; 1= at targeted breath alcohol; 2, 3= between each of the three BOLD scans; 4=following the third (last) BOLD scans.

*Significant main effect of genotype ($p < 0.05$).

Figure 2. Craving

Desire to drink (craving) at baseline (prior to any stimulus exposure) and following combined odor and picture presentation outside the scanner room and just prior to imaging (see text for abbreviations). *Significantly different from baseline ($p < 0.001$).

Figure 3. Medial Prefrontal Activation [AO > ApCO] Effects

All effects shown are collapsed across alcohol and placebo. **A.** Location of peak effects as obtained in an SPM factorial model of the [alcohol odor (AO) > appetitive odor control (ApCO)] effect (display threshold, $p < 0.005$, extent threshold = 25 voxels) and their overlap with the *a priori* regions of interest (blue= mPFC, green= vmPFC). **B.** Peak locations for significant group (AA > AG) effects of the [AO > ApCO] BOLD contrast (top= peak voxel effects; bottom= nature of interaction within ROIs). Display threshold as in panel A. mPFC= medial prefrontal cortex; vmPFC= ventromedial prefrontal cortex; x= lateral coordinate in stereotactic space; BOLD= blood oxygen level dependent.

Figure 4. VTA Activation [AO > ApCO] Effects

Peak ventral tegmental area (VTA; green dashed circle) location of the [alcohol odor (AO) > appetitive odor control (ApCO)] response (collapsed across alcohol and placebo), where AG subjects activate more than AA subjects. Display threshold, $p < 0.005$, extent threshold = 0. BOLD= blood oxygen level dependent.

Table 1

Subject Characteristics

	AA genotype <i>n</i> = 13 [*]			AG genotype <i>n</i> = 23			<i>p</i> [†] =
	Mean	(SD)	<i>n</i> (%)	Mean	(SD)	<i>n</i> (%)	
Age	23.15	(2.15)		24.26	(2.71)		0.22
Male			7 (54%)			14 (61%)	0.20
Caucasian			13 (100%)			18 (78%)	0.35
Education (years)	14.85	(1.68)		15.43	(1.04)		0.27
Family History Positive			4 (31%)			8 (35%)	0.81
Num. of Relatives w/Alcoholism	1.08	2.06		0.96	(1.43)		0.84
Recruited as Social Drinker			3 (23%)			10 (43%)	0.22
Num. Drinks/Week	14.98	(12.43)		12.45	(8.14)		0.46
Num. Drinks/Drinking Day	4.44	(1.94)		4.10	(2.20)		0.65
Num. Heavy Drinking Days/Week	1.32	(1.08)		1.08	(0.95)		0.50
AUDIT	10.62	(5.03)		8.87	(5.02)		0.32
Age at First Drink	15.62	(1.76)		16.30	(2.44)		0.38
Age of Regular Drinking	18.92	(1.66)		19.17	(1.59)		0.66
Smokers			4 (31%)			4 (17%)	0.35

Notes. AUDIT= Alcohol Use Disorder Identification Test. Drinking frequency data derived from the Timeline Followback Interview. Family history positive = two or more first or second degree relatives with probable alcoholism.

* All 13 AA subjects' fMRI data were available for analysis in the placebo session; 11 AA subjects' fMRI were available for analysis in the alcohol session; see text.

[†] Significance of group differences (*t*-test or χ^2 as appropriate).