# The Role of *GABRA2* in Alcohol Dependence, Smoking, and Illicit Drug Use in an Australian Population Sample

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**Background:** Multiple studies have shown that genetic variation in the α-2 subunit of the gamma-aminobutyric acid type A ($GABA_A$) receptor (GABRA2) is associated with risk for alcohol dependence. Recent reports have suggested that *GABRA2* may exert its influence on dependence through factors such as sensitivity to alcohol's intoxicating effects and that *GABRA2* may also contribute to a common underlying genetic vulnerability to both alcohol and drug dependence. The present study tested for association between *GABRA2* and alcohol dependence, smoking, and illicit drug use within the Australian population.

**Methods:** We genotyped 11 single nucleotide polymorphisms (SNPs) within or flanking *GABRA2* in 4597 subjects (34.6% males) from 2618 families comprising 814 monozygotic pairs, 1177 dizygotic pairs, and 627 twins whose co-twin did not participate. Family-based association tests were conducted for binary and quantitative measures of alcohol dependence, smoking, and cannabis and other illicit drug use.

**Results:** We observed evidence of association ($p < 0.05$) between multiple *GABRA2* SNPs and quantitative measures of alcohol dependence, including symptom scores and principal component factor scores from the 9 criteria for DSM-IV alcohol dependence, in the opposite direction to that previously reported. In contrast, *GABRA2* was not associated overall with dichotomous measure of alcohol dependence nor with smoking, cannabis, or illicit drug use.

**Conclusions:** The *GABRA2* allelic associations found in clinical case–control studies have detectable but minor effects on DSM-defined alcohol dependence in the general community. Systematic comparisons of allelic effects on alcohol dependence in clinical cases and in the general community are required.

**Key Words:** Alcoholism, Cannabis, Drug Dependence, GABRA2, Smoking.

A COMBINATION OF suggestive linkage findings (Long et al., 1998; Porjesz et al., 2002; Reich et al., 1998; Williams et al., 1999) and biological plausibility (Buck, 1996; Davies, 2003; Grobin et al., 1998; Koob, 2004; Krystal et al., 2006) led the Collaborative Study on the Genetics of Alcoholism (COGA) to conduct the first fine-mapping association study of the gamma-aminobutyric acid type A ($GABA_A$) receptor (GABR) gene cluster, investigating of alcohol dependence (AD) and β-electroencephalogram activity in a cohort of U.S. families enriched for alcoholism (Edenberg et al., 2004). The GABR gene cluster on chromosome 4 comprises 4 genes (*GABRG1*, *GABRA2*, *GABRA4* and *GABRB1*) where the strongest linkage finding in relation to the AD phenotype was detected with markers (D4S3242, D4S2393) close to *GABRB1* (Long et al., 1998; Williams et al., 1999). The family-based COGA study genotyped 69 single nucleotide polymorphisms (SNPs) across the gene cluster and only detected significant allelic and haplotypic association with SNPs in *GABRA2,* where the region of strongest association with AD extended from intron 3 past the 3′ end of *GABRA2*. A recent study by Covault et al. (2008) has extended the observed association from the *GABRA2* locus to potential regulatory regions immediately upstream of *GABRG1* (downstream of *GABRA2*). Independent studies have confirmed the association between *GABRA2* and AD in other Caucasian populations from the United States (Covault et al., 2004), Russia (Lappalainen et al., 2005), and Germany (Fehr et al., 2006; Soyka et al., 2008) using a case–control approach, while other groups have found no association (Drgon et al., 2006; Matthews et al., 2007).

With respect to mechanisms for this effect, Pierucci-Lagha et al. (2005) and Haughey et al. (2008) have proposed that increased risk for AD may be because of *GABRA2* moderating the subjective effects of alcohol (an individual's susceptibility to intoxication). Other evidence suggests that *GABRA2* may contribute to a common underlying genetic vulnerability to alcohol and drug dependence. It is known from twin

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studies that there are shared genetic risk factors between alcohol and nicotine dependence (Madden et al., 2000; True et al., 1999) as well as alcohol and drug dependence (Kendler et al., 2003).

The COGA group recently reported an association between *GABRA2* and drug dependence, including illicit drugs and cannabis (Agrawal et al., 2006). Importantly, subsequent analysis of the COGA dataset showed that the initial association between *GABRA2* and AD was primarily driven by those individuals with co-occurring illicit drug dependence and when those individuals were excluded, evidence for association with AD disappeared (Agrawal et al., 2006). In contrast, excluding European-American AD cases comorbid for drug (cocaine and/or opioid) dependence increased the strength of association between *GABRA2* and AD (Covault et al., 2004). However, while Drgon et al. (2006) did not find association between 6 *GABRA2* SNPs and AD in their European-American case and control sample, they did observe a trend towards significance ($p = 0.052$) with polysubstance abuse among African-Americans.

Other studies have revealed further complexity in the relationship between *GABRA2* and AD risk where *GABRA2* modulates risk for other co-occurring psychiatric disorders including conduct disorder (Dick et al., 2006) and anxiety (Enoch et al., 2006) and the presence of comorbidity within AD samples has been shown to affect the likelihood of observing an association between *GABRA2* and AD. However, the interaction can result in conflicting findings. While Covault et al. (2004) observed stronger association with AD in a restricted sample of alcoholics without comorbid major depression, Matthews et al. (2007) found no evidence of association between *GABRA2* and AD in alcoholic subjects selected for minimal primary comorbidity for drug dependence and other psychiatric disorders.

In the present study we primarily test for association between 11 SNPs spanning *GABRA2* and AD and alcohol consumption measures in a sample of 4597 individuals representative of the general Australian population. With data available on tobacco, cannabis, and illicit drug use measures, we then expanded our analyses to test whether the pathway from *GABRA2* genetic variation to alcohol-related outcomes may also modulate risk for these frequently co-occurring substance use disorders.

## MATERIALS AND METHODS

### *Participants*

Participants were recruited from 1992 to 1995 for a telephone interview-based twin study conducted at the Queensland Institute of Medical Research (QIMR; Heath et al., 1997). This interview was based upon an Australian modified version (SSAGA-OZ) of the Semi-Structured Assessment for the Genetics of Alcoholism instrument (SSAGA; Bucholz et al., 1994) designed for genetic studies of alcoholism. The SSAGA is a psychiatric interview which retrospectively assesses physical, psychological, and social manifestations of AD along with several other psychiatric disorders (American Psychiatric Association, 1994) and has undergone both reliability and validity testing (Bucholz et al., 1994; Hesselbrock et al., 1999). A total of 4597 subjects (34.6% males) from 2618 families comprising 814 (583 female and 231 male) monozygotic (MZ) pairs, 1177 (482 female, 198 male, and 491 opposite sex) dizygotic (DZ) pairs, and 627 twins (38.8% male) whose co-twin did not participate were included in genetic analysis. The participants were predominantly of North-European ancestry (>90%) and aged 26 to 89 years (mean age was 43.8 ± 11.5 years) at the time of testing. Subjects gave written informed consent and provided blood samples from which DNA was isolated using standard protocols. Genetic studies were approved by the QIMR Human Research Ethics Committee. Zygosity of same sex twins was assessed using 9 polymorphic DNA microsatellite markers (AmpF1STR Profiler Plus Amplification Kit, Applied Biosystems, Foster City, CA) and 3 blood groups (ABO, MNS, and Rh), giving a probability of correct assignment greater than 99.99% (Nyholt, 2006a).

### *Phenotypes*

*Alcohol*. Diagnostic assessment of lifetime history of AD was ascertained from the adapted SSAGA interview data. AD was diagnosed by computer algorithms based on the criteria of the Third Revised (DSM-IIIR) and Fourth (DSM-IV) Diagnostic and Statistical Manuals of the American Psychiatric Association (American Psychiatric Association, 1987, 1994). Similarly, DSM-IV alcohol abuse was assigned by computer algorithm. In addition to the dichotomous definition of AD, more informative (quantitative) measures were calculated and a summary of the alcohol-related traits examined in this study is given in Table 1.

1. Number of criteria met for DSM-IIIR (sumAD3r) and DSM-IV (sumAD4) AD.
2. Principal component factor analysis of the 7 criteria for DSM-IV (1 factor with an eigenvalue >1; factAD4) and 9 criteria for DSM-IIIR (2 factors had an eigenvalue >1; factAD3r_1 and factAD3r_2): the factAD4 factor accounted for 37.7% of the variance in the symptom count variable while the factAD3r_1 and factAD3r_2 scores accounted for 34.5 and 13.0%, respectively.
3. *Alcohol consumption*: frequency and quantity of alcohol consumption; maximum number of alcoholic drinks consumed in a single day ever and in a single day within the past 12 months.

*Cigarette Smoking*. The SSAGA did not assess smoking-related measures, but information on smoking-related phenotypes were available from a Health and Lifestyle Survey mailed questionnaire that the twins responded to in 1989 (Whitfield et al., 2000). Two smoking-related measures were coded.

1. *Current smoking*. A dichotomous item reflecting whether the participant was a current smoker at the time of questionnaire assessment, with never smokers set to missing;
2. *Pack years of cigarettes smoked*. A quantitative measure calculated as: Pack years = (cigarettes smoked on an average day × total years smoked)/20; the number of cigarettes smoked on an average day was calculated from a categorical variable ranging from 1 (never smoked) to 6 (smoked 40+ cigarettes per day).

*Illicit Drug Use*. Diagnostic criteria for abuse and dependence were not included in the SSAGA interview. Therefore, we used the following assessments of illicit drug use.

1. *Quantitative cannabis use*. An ordinal measure of cannabis use was created from the participant's report of how often in their entire lifetime they had used cannabis. Participants could respond with any number, but were also given the option of responding "Too many times to count" which was assumed to be greater than 950. These responses were then coded into a 5-category ordinal scale, defined separately for males and

**Table 1.** Summary of the Sample Demographics and Alcohol-Related Traits by Sex

| Item | Assessment | Male | Female |
|---|---|---|---|
| Sample | Number of participants, by sex | 1592 | 3005 |
| Age | Age at time of interview (years) (age range, years) | 42.6 ± 10.9 (26–89) | 44.3 ± 11.8 (28–84) |
| Abstainer | Has never consumed alcohol | 36 (2.2%) | 87 (2.9%) |
| Current | Has consumed alcohol in the previous 12 months | 1474 (92.6%) | 2784 (92.6%) |
| Freq | Drinking frequency during the past 12 months (days) | 124.6 ± 112.8 | 82.7 ± 105.2 |
| | Percentage who drink on a daily basis | 7.6% | 5.5% |
| | Percentage who drink on a weekly basis | 67.0% | 44.9% |
| TypDy | Number of drinks typically consumed on days when they drank alcohol in the past 12 months (percentage of men who consume >6 drinks and women who consume >3 drinks) | 2.9 ± 2.3 (4.0%) | 1.9 ± 1.5 (9.5%) |
| Quant | Quantity of drinks consumed in previous 12 months (Freq*TypDy) | 406.9 ± 537.8 | 181.1 ± 277.3 |
| MaxDr12 | Most drinks consumed in a single day in the previous 12 months | 7.6 ± 6.6 | 3.7 ± 3.2 |
| MaxDr | Most drinks ever consumed in a single day | 16.7 ± 11.5 | 7.9 ± 6.0 |
| sumAbuse | Number of DSM-IV alcohol abuse criteria met (of four) | 0.55 ± 0.82 | 0.11 ± 0.40 |
| | Percentage with no symptoms | 61.4% | 90.8% |
| sumAD3r | Number of DSM-IIIR alcohol dependence criteria met (of seven) | 2.10 ± 2.12 | 0.78 ± 1.45 |
| | Percentage with no symptoms | 35.7% | 70.1% |
| sumAD4 | Number of DSM-IV alcohol dependence criteria met (of nine) | 1.30 ± 1.49 | 0.48 ± 0.96 |
| | Percentage with no symptoms | 39.6% | 71.4% |
| Abuse | Diagnosed with DSM-IV alcohol abuse | 584 (38.8%) | 267 (9.2%) |
| AD3r | Diagnosed with DSM-IIIR alcohol dependence | 375 (23.6%) | 172 (5.7%) |
| AD4 | Diagnosed with DSM-IV alcohol dependence | 290 (18.2%) | 131 (4.4%) |
| factAbuse | Single principal component factor score from DSM-IV alcohol abuse criteria | 0.42 ± 1.44 | −0.20 ± 0.62 |
| factAD3r_1 | First principal component factor score from DSM-IIIR alcohol dependence criteria | 0.43 ± 1.26 | −0.20 ± 0.76 |
| factAD3r_2 | Second principal component factor score from DSM-IIIR alcohol dependence criteria | 0.45 ± 1.24 | −0.21 ± 0.77 |
| factAD4 | Single principal component factor score from DSM-IV alcohol dependence criteria | −0.28 ± 1.28 | 0.13 ± 0.81 |

females. For males these categories were 1 to 2 times, 3 to 9, 10 to 49, 50 to 499, and 500+; for females they were 1, 2 to 4, 5 to 11, 12 to 99, and 100+. Frequency was also coded in all participants, including lifetime abstainers who were coded as 0.

2. *Other illicit drug use*. A dichotomous item reflecting whether the participant had used an illicit drug other than cannabis, including cocaine, stimulants, sedatives, opiates, solvents, hallucinogens, phencyclidine, or other illicit drugs, even once in their lifetime.
3. *Drug problems*. A dichotomous item assessing severe liability to drug abuse/dependence was coded positively if the respondent said that they had: "… ever discussed any other drug problem with any professional" or if they had "… ever been treated for a drug problem."

### *Genotyping*

Fourteen SNPs across the *GABRA2* locus were selected on the basis of previous work completed by our group (Lind et al., 2008). Briefly, 41 SNPs within or flanking *GABRA2* were genotyped in an independent Australian twin sample (Alcohol Challenge Twin Study) that completed an alcohol challenge test (Martin et al., 1985). The 41 SNPs were selected on the basis of data available at the time from (i) association studies (e.g., Covault et al., 2004; Edenberg et al., 2004; Pierucci-Lagha et al., 2005) and (ii) the International HapMap Project public database (*http://www.hapmap.org/*; Phase II dbSNP Build 124). Linkage disequilibrium (LD) data from this study indicated that a reduced set of 14 haplotype-tagging SNPs would provide appropriate coverage ($r^2 \geq 0.95$) of *GABRA2*. Three SNPs (rs279871, rs279836, rs497068) failed during the assay design or provided unreliable genotype data and were excluded. The locations of the remaining 11 SNPs typed in the study are shown in Fig. 1 and SNP information, including the observed minor allele frequency, is described in Table 2.

Genotyping forward and reverse polymerase chain reaction (PCR) primers and an extension primer were designed using the Sequenom MassARRAY Assay Design (version 3.0) software (Sequenom Inc., San Diego, CA) and purchased from Bioneer Corporation (Daejeon, Korea). Genotyping was carried out in standard 384-well plates with 12.5 ng genomic DNA used per sample. We used a modified Sequenom protocol where half reaction volumes were used in each of the PCR, shrimp alkaline phosphatase, and iPLEX stages giving a total reaction volume of 5.5 $\mu$l. The iPLEX reaction products were desalted by diluting samples with 18 $\mu$l of water and 3 $\mu$l SpectroCLEAN resin (Sequenom Inc.) and then were spotted on a SpectroChip (Sequenom Inc.), processed and analyzed on a Compact MALDI-TOF Mass Spectrometer by MassARRAY Workstation software (version 3.3) (Sequenom Inc.). Allele calls for each 384-well plate were reviewed using the cluster tool in the SpectroTyper software (Sequenom Inc.) to evaluate assay quality. Genotype error checking, sample identity, and zygosity assessment and Hardy–Weinberg equilibrium (HWE) analyses were completed in PEDSTATS (Wigginton and Abecasis, 2005).

### *Statistical Analyses*

*Association Analyses*. Quantitative trait analysis was performed in QTDT (Abecasis et al., 2000a,b), with 22 traits examined. All alcohol-related quantitative traits were first transformed to

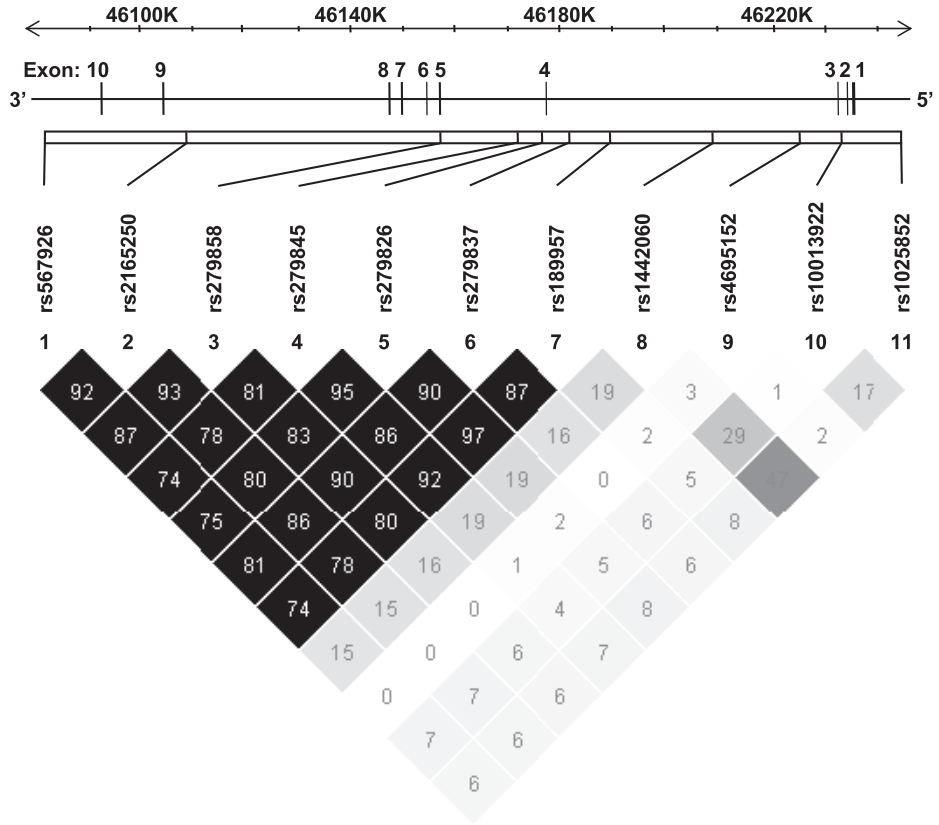


**Fig. 1.** A schematic representation of gamma-aminobutyric acid type A receptor (GABRA2) gene structure, linkage disequilibrium (LD), and genotyped markers. The gene structure of *GABRA2* is shown with exons numbered and relative exon size denoted by the width of the vertical bars. The 11 single nucleotide polymorphisms analyzed in this study are shown in relation to their location across *GABRA2* and regions of low to high pairwise LD, as measured by the $r^2$ statistic, are represented by light gray to black shading, respectively.

**Table 2.** *GABRA2* Marker Information, Including Genetic Map Position, Location Within *GABRA2*, and Minor Allele Frequency

| | | | | | Alleles | | |
|---|---|---|---|---|---|---|---|
| SNP No. | SNP | Chromosomal location[a] | Functional location[b] | Gene position | Minor[c] | Major | MAF |
| 1 | rs567926 | 45,936,526 | 150,176 | 3′UTR | C | T | 0.418 |
| 2 | rs2165250 | 45,963,241 | 123,461 | Intron 8 | C | T | 0.420 |
| 3 | rs279858 | 46,009,350 | 77,352 | Exon 5 | G | A | 0.433 |
| 4 | rs279845 | 46,024,480 | 62,222 | Intron 4 | T | A | 0.435 |
| 5 | rs279826 | 46,028,966 | 57,736 | Intron 4 | C | T | 0.452 |
| 6 | rs279837 | 46,034,080 | 52,622 | Intron 3 | C | T | 0.432 |
| 7 | rs189957 | 46,041,436 | 45,266 | Intron 3 | C | T | 0.449 |
| 8 | rs1442060 | 46,060,824 | 25,878 | Intron 3 | T | C | 0.489 |
| 9 | rs4695152 | 46,076,414 | 10,288 | Intron 3 | C | G | 0.039 |
| 10 | rs10013922 | 46,084,118 | 2,584 | Intron 2 | C | A | 0.261 |
| 11 | rs1025852 | 46,095,088 | −8,386 | Promoter | T | C | 0.368 |

MAF, minor allele frequency; SNP, single nucleotide polymorphism; UTR, untranslated region.
[a]Position in nucleotides as estimated in dbSNP (Build 128).
[b]Position relative to the transcription start site at 46,086,702 on chromosome 4 (dbSNP Build 128).
[c]The allele with the lowest frequency.

normality using a piecewise normal transformation and were corrected for sex and age effects by fitting covariates in the regression model. Drug-related quantitative traits (i.e., pack years and quantitative cannabis use) were log-transformed, the effects of sex, age (linear and quadratic), and interactions between sex and age (linear and quadratic) were tested in a regression framework, significant covariates were regressed out and the resulting residuals were used for quantitative transmission disequilibrium tests. Three types of association tests were performed. An analysis robust to population stratification was conducted using the orthogonal "within" test in QTDT. As parents were not genotyped, this analysis only included families with 2 DZ offspring. To account for the fact that transmissions were not independent in the DZ families because of the presence of linkage in DZ pairs, first, a permutation procedure (10,000 permutations) was used to correct the *p*-values for the orthogonal test. Second, analyses of all 2618

families were performed using a test of total association which considers transmissions between and within families. For the test of total association, families with either 1 twin or 2 MZ twins only contribute to the "between" component and a simple permutation procedure cannot be applied. Thus, nonindependent transmissions were corrected by modeling linkage and association within a maximum likelihood framework. MZ twin status was included in QTDT analyses by adding zygosity status to the data file. Third, quantitative haplotypic association was tested in the QPDTPHASE module of the UNPHASED statistical package (Dudbridge, 2003) which only allows analysis of the "within" component in DZ families. To reduce multiple testing, haplotype testing was only performed on 4 alcohol-related traits of interest (factAD3r_1, factAD4, sumAbuse, sumAD3r).

Binary trait analysis was performed for comparison with the above quantitative trait analysis and also for comparison with the work of other groups. Association of DSM-IIIR and DSM-IV AD was examined using the UNPHASED statistical package in 2 stages. First, for DZ families, a pedigree disequilibrium (PDT) style test was used in the PDTPHASE module to test for overtransmission of allele to offspring (a "within" family test that should be robust to any population stratification effects). Second, for MZ families, pairs with discordant phenotypes were set to phenotype unknown while pairs with concordant phenotypes were not changed. Subsequently, 1 MZ twin per family was included for each trait analyzed in COCAPHASE. As no parents were genotyped, each MZ twin was treated as unrelated and a case–control test was implemented.

Pairwise marker–marker LD was assessed using the D′ and $r^2$ statistic in HAPLOVIEW 3.31 (Barrett et al., 2005). Many of the traits studied were correlated and there was substantial LD across *GABRA2* [see (Fig. 1)]. As a result the effective number of statistical tests carried out was substantially less than the actual number of tests. To take account of both of these factors, we estimated the effective number of tests by utilizing permutations in QTDT. As QTDT only implements permutations for the "within" test, we cannot do this for the "total" test (but see below). Two *p*-values are available from the permutation routine; one which corrects for all tests carried out, $p_{\text{corrected}}$ and another *p*-value for each trait/marker combination, $p_{\text{uncorrected}}$. If all traits and markers were uncorrelated then a permutation procedure would yield a *p*-value equivalent to a Bonferroni correction; that is, $p_{\text{corrected}} = 1 - (1 - p_{\text{uncorrected}})\hat{}n$ where $n$ denotes the number of tests carried out. An estimate of the effective number of independent "within" tests is hence $n_{\text{effective}} = \log(1 - p_{\text{corrected}})/\log(1 - p_{\text{uncorrected}})$, approximately 110 (compared with 242 nonindependent tests in total). Making the simplifying assumptions that (i) the "total" tests exhibit similar correlation between tests as the "within" test and (ii) that the "within" and "total" tests are independent, the total number of effectively independent tests is *c*. 220. As the "within" and "total" tests are in fact correlated, the most appropriate correction for multiple testing based on the "effective number of independent tests" would be correction between 110 and 220 tests. That is, a *p*-value smaller than 0.00045 (and perhaps as small as 0.000225) is required for study wide significance.

*Survival Analyses*. Cox proportional hazards models were set up in STATA (StataCorp LP, College Station, TX; StataCorp, 2003). Twin clustering was accounted by allowing members of a twin pair to cluster on their family number, which allows standard errors to be adjusted using a robust variance estimator. The event of interest for the survival analysis was one or more symptoms of AD, and age of onset of the first DSM-IIIR AD symptom was the time to event. Individuals who had never used alcohol even once in their lifetime and those who used alcohol, but at the time of interviews reported no dependence symptoms and were censored and their time to event was set to their age at interview.

The hazard ratios for the association between genotype and age at first symptom of AD were computed in a univariate model and then in a model that adjusted for sex as well as an interaction between sex and genotype. Genotypes that were significantly associated with AD symptoms in QTDT analyses (rs279858 and rs279845) were selected for the survival analyses. For the first series of analyses (Models 1–3), genotype was coded as 0 (no copies of risk allele from QTDT), 1 (1 copy of risk allele from QTDT), and 2 (2 copies of risk allele from QTDT). In a second series of analyses (Models 4–6), genotype was coded under a dominance model as 0 (no copies) and 1 (1 or 2 copies of risk allele). In models 1 and 4, only genotype coded as 0, 1, and 2 and 0 and 1, respectively, was entered into the model. In models 2 and 5, both genotype and sex were included while in models 3 and 6, the effects of genotype, sex, and genotype*sex were examined.

## RESULTS

### *Descriptive*

A total of 4597 twin participants provided blood samples and completed the SSAGA-OZ questionnaire. A total of 2618 families were included in the analysis. As parents were not genotyped, only families containing a nonidentical sibling pair contributed to the "within" test of association. A total of 1177 families included a DZ pair and hence were potentially informative for both the "within" and the "total" test of association. A total of 814 families included an MZ pair and hence contributed information to just the "total" test. Similarly, 627 families had just a single offspring (i.e. trios) and these contributed information to just the "total" test. The mean age of the study population was 43.8 ± 11.5 years, ranging from 26 to 89 years. The mean age of female participants was significantly higher (44.3 vs. 42.6) than for males ($p = 0.001$) (see Table 1). While a small percentage (< 3%) of the sample had always abstained from alcohol, the majority (*c*. 93%) had consumed alcohol in the previous year, with 7.6 and 5.5% of male and females, respectively, reporting that they drank alcohol on a daily basis. With respect to AD, male participants were approximately threefold more likely to meet a lifetime diagnosis of either DSM-IIIR (23.6%) or DSM-IV (18.2%) dependence than female twins. However, *c*. 37% of males and 70% of females did not report any symptoms of AD. Similarly, most twins did not meet lifetime criteria for DSM-IV alcohol abuse.

In our sample, less than half of the participants (44.5% of women and 36.4% of men) who have ever smoked reported current smoking at the time of the interview. The mean pack years smoked was 12.7 [range: 0–97] and 15 [range: 0–89] in women and men, respectively. Additionally, 19.4 and 34.7% of the women and men, respectively, reported lifetime cannabis use while 12.7% of women and 16.9% of men reported use of other illicit drugs. The mean lifetime number of times that the users had consumed cannabis was 19.5 and 50.6 in the women and men, respectively.

Marker information including genetic map position, location within *GABRA2*, and the minor allele frequencies of the 11 SNPs genotyped within our Australian twin sample are listed in Table 2. Call rates of ≥98% were achieved for all SNPs except rs567926 where 96.9% of the sample was successfully genotyped. No SNPs showed significant deviations

from HWE at a $p < 0.01$ level. Discordant genotypes between MZ twins were identified using PEDSTATS and made up for 0.12% of the data. The physical locations of, as well as LD between, the 11 SNPs typed across the *GABRA2* gene are presented schematically in Fig. 1. Substantial LD was observed between 7 markers spanning from intron 3 to the untranslated region (3′UTR), with lower levels of LD ($r^2 < 0.3$) observed 5′ to this major haplotype block. This is consistent with LD data (on a smaller number of people) from the HapMap database for Centre d'Etude du Polymorphisme Humain families of European origin.

### *Single SNP Association Analyses*

*Alcohol-Related Measures.* Within-family association results utilizing 10,000 permutations for 2 quantitative measures of DSM-IV AD are reported in Table 3. The strongest evidence of association was found for the synonymous SNP rs279858 located in exon 5 ($p = 0.007$), with the G allele conferring a lower DSM-IV factor score (factAD4). With a correlation of 0.978 between the DSM-IV factor score and the number of DSM-IV AD symptoms experienced (sumAD4), the G allele also reduced the sumAD4, where the average number of symptoms reported in the study were 0.78, 0.76, and 0.71 in individuals with 0, 1, and 2 G-alleles, respectively. The T-allele of rs279845 also conferred lower factAD4 and sumAD4 scores. The directions of effect for both rs279858 and rs279845 are contrary to that reported in previous studies (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005). Three other SNPs located in intron-3 and intron-4 were nominally associated with the factAD4 and sumAD4 measures. Similar levels of association ($p < 0.05$) were observed for four of the SNPs with the closely related "sum of DSM-IIIR AD symptoms" phenotype (data not shown). The "total" association results for the quantitative measures shown in Table 3 were nonsignificant.

**Table 3.** Permutation-Based *GABRA2* "Within" Association Results for 2 Quantitative Measures of DSM-IV Alcohol Dependence

| | | | *p*-value[a] | |
|---|---|---|---|---|
| SNP No. | SNP | Gene position | sumAD4 | factAD4 |
| 1 | rs567926 | 3′UTR | 0.21 | 0.18 |
| 2 | rs2165250 | Intron 8 | 0.16 | 0.12 |
| 3 | rs279858 | Exon 5 | 0.01 | 0.007 |
| 4 | rs279845 | Intron 4 | 0.01 | 0.01 |
| 5 | rs279826 | Intron 4 | 0.02 | 0.02 |
| 6 | rs279837 | Intron 3 | 0.11 | 0.10 |
| 7 | rs189957 | Intron 3 | 0.03 | 0.03 |
| 8 | rs1442060 | Intron 3 | 0.04 | 0.04 |
| 9 | rs4695152 | Intron 3 | 0.52 | 0.80 |
| 10 | rs10013922 | Intron 2 | 0.24 | 0.24 |
| 11 | rs1025852 | Promoter | 0.52 | 0.45 |

factAD4, single principal component factor score from DSM-IV alcohol dependence criteria; SNP, single nucleotide polymorphism; sumAD4, number of DSM-IV alcohol dependence criteria met; UTR, untranslated region.

[a]*p*-values reported after 10,000 permutations run to correct for family structure.

Although the "within" association is not significant after taking account of multiple testing (with a threshold level of significance set at 0.00045, see Materials and Methods), where multiple studies reporting association between *GABRA2* and AD have been published. Our results provide limited support for a role of *GABRA2* in risk for alcoholism.

Finally, while the correlation between sumAD3r and factAD3r_1 is strong (0.911), the correlation falls to 0.370 with the second factor score (factAD3r_2) which primarily loads onto the 2 DSM-IIIR criteria that address alcohol withdrawal. We did not observe an association with factAD3r_1 and only detected nominal association ($p = 0.03$) between one SNP in intron-3 (rs4695152) and the second factor score. Previous association studies have found evidence that *GABRA2* genetic variation modulates aspects of alcohol withdrawal (Fehr et al., 2006; Soyka et al., 2008) and this relationship may explain, to an extent, our finding with the factAD3r_2 score. For both "within" and "total" tests, no significant association (smallest *p*-value *c*. 0.01 before correction for multiple testing) was detected between the alcohol use measures, including quantity and frequency of consumption, and SNPs in *GABRA2*.

Subsequent binary analyses of DSM-IIIR and DSM-IV AD (AD3r, AD4) diagnoses were run in DZ families to permit within-family tests of association to be performed. The purpose of these analyses was to be able to directly compare our findings with those of other groups who only analysed the dichotomous diagnoses of AD. The number of affected (case) individuals in the entire sample is as follows: AD3r (959 cases) and AD4 (421). Within DZ families only, however, the number was reduced to 540 (AD3r) and 224 (AD4) cases; we did not observe significant association between binary measure and the 5 SNPs of interest identified previously. Similarly, we did not observe any association with AD3r or AD4 in our MZ sample of unrelated cases and controls.

*Smoking and Drug Use Measures.* For both log-transformed pack years and quantitative cannabis use, sex and age were significantly associated and consequently were regressed out. Men reported greater smoking in pack years ($\beta = -0.11$, SE = 0.05) and a higher frequency of cannabis use ($\beta = -0.14$, SE = 0.02) while older individuals reported more pack years smoked ($\beta = 0.03$, SE = 0.002) but reported less cannabis use ($\beta = -0.14$, SE = 0.02). For the binary traits, with the exception of self-reported history of drug-related problems ($n = 2$ affected MZ twins), we were able to use both DZ pairs and a single MZ from a concordant affected pair (in a case–control framework) to test for association. For current smoking status, 270 DZ (89 concordant affected) pairs and 176 (84 concordant affected) representative members from a concordant MZ pair were available. No significant association was noted in the within-family analysis of smoking or illicit drug use measures and SNPs in *GABRA2*. However, in the MZ only sample of unrelated cases and controls, current smoking status in people who have ever smoked was associated with 7 SNPs in *GABRA2* (see

**Table 4.** *GABRA2* Association Results for Current Smoking Status Using DZ Sibling Pairs (PDTPHASE) and Unrelated Cases and Controls From MZ Pairs

| | | | *p*-value | |
|---|---|---|---|---|
| SNP No. | SNP | Gene position | DZ siblings | Case–control (from MZ pairs) |
| 1 | rs567926 | 3′UTR | 0.69 | 0.02 |
| 2 | rs2165250 | Intron 8 | 0.89 | 0.04 |
| 3 | rs279858 | Exon 5 | 0.89 | 0.02 |
| 4 | rs279845 | Intron 4 | 0.29 | 0.02 |
| 5 | rs279826 | Intron 4 | 0.69 | 0.007 |
| 6 | rs279837 | Intron 3 | 0.79 | 0.007 |
| 7 | rs189957 | Intron 3 | 0.79 | 0.009 |
| 8 | rs1442060 | Intron 3 | 0.66 | 0.23 |
| 9 | rs4695152 | Intron 3 | 0.32 | 0.27 |
| 10 | rs10013922 | Intron 2 | 0.27 | 0.55 |
| 11 | rs1025852 | Promoter | 0.99 | 0.56 |

DZ, dizygotic; MZ, monozygotic; SNP, single nucleotide polymorphism; UTR, untranslated region.

Table 4), the same that were associated with quantitative measures of AD in Table 3. Our most significant finding was with rs279826 ($p = 0.007$), however in the DZ only analyses, the corresponding *p*-value for this analysis was $p = 0.69$.

### *Haplotypic Association Analyses*

Using a sliding window method, a series of 2- and 3-SNP haplotypes were analyzed in 3 quantitative phenotypes: factAD4, factAD3r, and sumAbuse (the 1177 DZ families were used for this analysis). For factAD4, the smallest *p*-value for any 2-SNP haplotype was between rs279858 and rs279845 ($p = 0.01$, uncorrected for multiple testing). This is no better than the best single marker result ($p = 0.007$). Haplotypes for all the other variables produced less significant results than for the single markers. Haplotype association analysis between sumAD3r and the LD block spanning SNPs 1 to 7 (Table 2) was not significant, with 2 complementary common (>5%) haplotypes observed: T-T-**A-A**-T-T-T (59.1%), C-C-**G-T**-C-C-C (36.5%) where the rs279858 and rs279845 alleles are highlighted in bold.

Because of the association between current smoking status and SNPs in *GABRA2*, we also conducted haplotype tests on the unrelated MZ case–control sample (dropping haplotypes with frequencies <0.01). Sliding windows revealed the most significant 2-SNP haplotype ($p = 0.01$) to be between rs2165250 and rs279858 with overtransmission of T-G (64%) to the controls. Haplotype association with the 7-SNP haplotype showed evidence of overtransmission of the T-T-G-**A-T**-T-T (63%) to the controls/ex-smokers and of the complementary C-C-A-**T-C**-C-C (47%) to the cases/current smokers was noted (global *p*-value of 0.03) where the rs279845 and rs279826 alleles are highlighted in bold.

### *Survival Analyses*

As described in the Materials and Methods section, the full set of twins was used in the survival analysis (4597 individuals). Two SNPs were selected for survival analyses: rs279858 and rs279845, our 2 strongest signals in the association analyses. For both rs279858 (A/G) and for rs279845 (A/T), A was the risk allele. With respect to rs279858, having 1 or more copies of the A allele was significantly associated with age at first AD symptom ($p = 0.014$) with A/A individuals at increased risk when compared with A/G individuals, and the G/G individuals at least risk (Fig. 2*A*). After adjustment for sex (Models 2 and 5 in Table 5), which was significantly associated with age at onset, genotype was still associated with age at onset. However, an interaction with sex was not significant (Models 3 and 6, which included the effects of genotype, sex, and their interaction in Table 5) and could be dropped from the model. An additive model fitted well—individuals with 2 copies of the risk allele were more likely to have an earlier age of onset of AD symptoms when compared with those carrying one copy of the risk allele.

While rs279845 was associated with age at onset of AD symptoms ($p = 0.02$), the greatest risk was noted for this with 2 copies of the risk allele (A/A) (Fig. 2*B*). Adjusting for sex reduced the effect of genotype. As shown in Fig. 2*B* and in Table 5, coding the genotype for a dominance model did

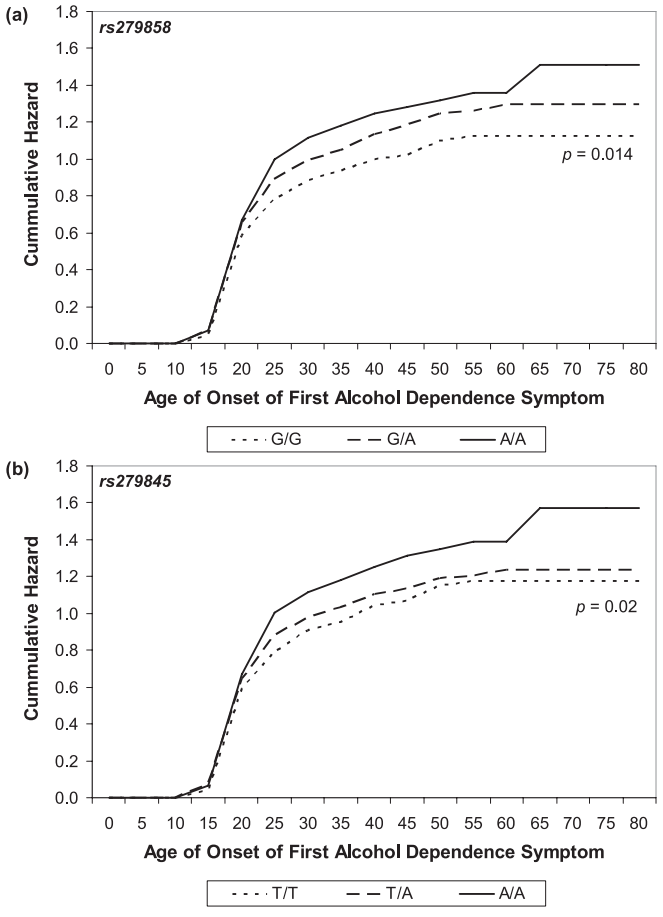


**Fig. 2.** Nelson-Aalen cumulative hazard estimates for age of onset of the first DSM-IIIR alcohol dependence symptom across the lifespan for (a) rs279858 and (b) rs279845 genotype. Tests of genotypic differences: (a) $p < 0.014$, (b) $p = 0.02$.

**Table 5.** Hazard Ratios for the Association Between 2 *GABRA2* Single Nucleotide Polymorphisms and Age at First Symptom of DSM-IIIR Alcohol Dependence

| | Single nucleotide polymorphism | | |
|---|---|---|---|
| Hazard model | rs279858 | rs279845 | rs279845 recessive |
| Model 1 | | | |
| Genotype (0/1/2) | 1.1 [1.02–1.19] | 1.1 [1.01–1.19] | |
| Model 2 | | | |
| Sex | 0.48 [0.43–0.53] | 0.47 [0.42–0.53] | |
| Genotype (0/1/2) | 1.09 [1.01–1.18] | 1.08 [1.00–1.16] | |
| Model 3 | | | |
| Sex | 0.44 [0.36–0.55] | 0.40 [0.32–0.49] | |
| Genotype (0/1/2) | 1.06 [0.96–1.18], ns | 1.01 [0.92–1.12], ns | |
| Sex*genotype (0/1/2) | 1.06 [0.91–1.22], ns | 1.14 [0.98–1.34], ns | |
| Model 4 | | | |
| Genotype (0/1) | 1.18 [1.03–1.36] | 1.14 [0.98–1.34], ns | 1.13 [1.01–1.27] |
| Model 5 | | | |
| Sex | 0.47 [0.42–0.53] | 0.47 [0.42–0.52] | 0.47 [0.42–0.52] |
| Genotype (0/1) | 1.19 [1.03–1.37] | 1.13 [0.98–1.30], ns | 1.09 [0.97–1.22], ns |
| Model 6 | | | |
| Sex | 0.46 [0.35–0.59] | 0.43 [0.33–0.55] | 0.43 [0.38–0.50] |
| Genotype (0/1) | 1.17 [0.96–1.42], ns | 1.07 [0.89–1.30], ns | 0.98 [0.84–1.15], ns |
| Sex*genotype (0/1) | 1.04 [0.78–1.39], ns | 1.12 [0.84–1.49], ns | 1.26 [1.0–1.58] |

ns, nonsignificant ($p > 0.05$).

not fit the data well. In contrast, a recessive model for genotype (A/A vs. A/T and T/T) fitted well. Interestingly, the interaction between genotype (recessive model) and sex was significant at $p = 0.05$ and rs279845 influenced age at onset of AD symptoms in women alone (data not shown).

## DISCUSSION

It is established that genetic variation in the alpha-2 subunit of the GABRA2 is associated with risk for AD (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005; Soyka et al., 2008). While the primary function of GABRs is to mediate the activity of GABA, the major inhibitory neurotransmitter in the central nervous system, GABRs also play a role in the chronic and acute effects of alcohol including motor incoordination, anxiolysis, motivation for excessive drinking, and alcohol withdrawal (Buck, 1996; Davies, 2003; Grobin et al., 1998; Koob, 2004; Krystal et al., 2006). Recently, *GABRA2* has also been shown to exert its influence on anxiety (Enoch et al., 2006) and several disinhibitory traits, such as conduct disorder (Dick et al., 2006), illicit drug dependence (Agrawal et al., 2006; Drgon et al., 2006) as well as nicotine dependence (Agrawal et al., 2008).

In the current study, we investigated the relationship between *GABRA2* and a series of quantitative and binary measures of alcohol consumption and dependence in a large twin sample representative of the general Australian population. Extending the scope of the study to incorporate other substance-use disorders that frequently co-occur with alcoholism, we then examined the role of *GABRA2* in smoking, cannabis, and other illicit drug use.

Consistent with previous findings, the strongest evidence for *GABRA2* SNP effects was with quantitative AD variables, namely, the number of DSM-IV criteria met for AD (sumAD4) and the principal component factor score extracted from the DSM-IV AD criteria variable (factAD4). Five SNPs, four of which are located within the major LD block that extends from intron 3 to the 3′UTR of *GABRA2* in our sample, were nominally associated ($p < 0.05$, "within" test of association) with these 2 phenotypes. The fact that this result is not significant after correction for multiple testing combined with the nonsignificant finding for these markers for the "total" test of association means that the effect of these markers on quantitative AD variables is modest at best.

Our most significantly associated SNP was the synonymous coding variant in exon 5 (rs279858) which has previously been found to be associated with AD status (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005) and subjective intoxication (Haughey et al., 2008; Pierucci-Lagha et al., 2005). In our data, the frequency of the rs279858 G allele was lower in DSM-IV diagnosed alcoholics (41.5%) compared with nonalcoholic twins (43.7%) and was, as expected, related to a lower DSM-IV factor score and number of DSM-IV AD symptoms experienced by each twin. The direction of effect is contrary to that reported by Pierucci-Lagha et al. (2005) where individuals carrying G alleles were less sensitive to the effect of alcohol and hence more likely to become alcohol dependent and in 3 studies where the G allele was significantly over-represented among alcoholics (Covault et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005). In each of these studies, the frequency of the G-allele ranged from 34% to 42% in the control samples—a frequency similar to that observed in our population. The G-allele has also been associated with an increased probability of daily drinking and heavy drinking during 12-week treatment and 12-month posttreatment periods in alcoholics participating in the Project MATCH study (Bauer et al.,

2007). Recently, Haughey et al. (2008) found that prefrontal cortex (PFC) *GABRA2* mRNA levels in postmortem brains and an individual's sensitivity to the acute affects of alcohol were significantly influenced by the *GABRA2* rs279858 SNP genotype. Heterozygous individuals exhibited less reward from alcohol than either homozygote, and postmortem brains of A/G individuals had significantly lower PFC mRNA levels compared with A/A homozygotes, suggesting that the A/G genotype may protect against developing AD.

The direction of effect for rs279845 was also contrary to that reported by Edenberg et al. (2004) and Fehr et al. (2006). While both studies reported an at-risk haplotype for AD composed of 3 SNPs (rs279871-rs279845-rs279836) there is some confusion given that the haplotype may differ with Edenberg et al. (2004) and Fehr et al. (2006) reporting T-A-T and C-T-T, respectively. However, a recent review of the role of GABRs in the development of AD indicates that the same (more frequent) haplotype was identified by Edenberg et al. (2004) and Fehr et al. (2006) (Enoch, 2008). While we did not successfully genotype rs279871 and rs279836, the high degree of LD spanning intron 3 to the 3′ end of *GABRA2* resulted in 2 common complementary haplotypes, accounting for 95.6% of the estimated chromosomes, and allows a comparison of previous studies with our rs279845 data given that the alleles are "A" and "T."

The at-risk rs279845 A-allele (along with the rs567926 T-allele, the at-risk rs279858 A-allele and the rs279837 T-allele) is carried on the more abundant haplotype in our population. In contrast, the at-risk (less frequent) C-C-G-T-T haplotype reported by Fehr et al. (2006) which includes rs567926 (C-allele), rs279858 (G) and rs279845 (T), and C-T-G-A-G-A-C haplotype identified by Covault et al. (2008) (including rs567926 C and rs279858 G), suggest we are observing an effect of the complementary haplotype on risk for AD in our population. Conversely, a recent case–control study of German treatment-seeking alcoholics, attempting to replicate the findings of Covault et al. (2004), identified the same (more frequent) at-risk haplotype as our study, observing T-A-T alleles at the rs567926, rs279858, and rs279837 loci (Soyka et al., 2008). Furthermore, the more frequent haplotype has been observed in Finnish alcoholics, albeit alcoholics with high tendency to be anxious (Enoch et al., 2006).

As *GABRA2* and another GABR gene (*GABRG1*) are in close proximity (128 Kb apart) with moderate LD reported to extend from the 3′ end of *GABRA2* to potential regulatory regions upstream of *GABRG1* (Covault et al., 2008), long-range LD between the 4 *GABRA2* SNPs of interest (rs279858, rs279845, rs279826, and rs189957) and variants in surrounding genes (including *GABRA4*, *GABRB1*) was investigated in ssSNPer (Nyholt, 2006b). In each analysis, only moderate LD (approximately $r^2 = 0.5$) was observed in the region between *GABRA2* and *GABRG1* loci, with lower levels ($r^2 < 0.3$) detected elsewhere, suggesting that the association signal is located within *GABRA2*.

None of the quantitative alcohol consumption measures showed significant associations, including the MaxDr and MaxDr12 phenotypes which have previously been shown to be highly correlated with alcohol use disorders (Saccone et al., 2000). Overall, there is little support for *GABRA2* allelic effects on alcohol intake in our population. Furthermore, *GABRA2* was not associated overall with smoking, cannabis, or other illicit drug use. However, evidence in a case–control subset suggested an association between *GABRA2* and current smoking status among ever-smokers (*i.e.*, smoking persistence), including the synonymous polymorphism, rs279858, which has recently been found to be associated with nicotine dependence in a sample of U.S. and Australian adults (Agrawal et al., 2008).

Significant SNP associations with current smoking were seen in the case–control sample derived from MZ twins, but not when using a Transmission Disequilibrium Test (TDT)-based analysis in the DZ sibling pairs. A reason for this may be the reduced power to detect association in the TDT framework when parental genotypes are missing and there is only one additional phenotyped (and genotyped) sibling (i.e. the DZ co-twin). Yang et al. (2003) demonstrated that for a common disease model, the absence of additional phenotyped siblings affects power—this limitation could extend to our analyses of alcohol and illicit drug-related phenotypes as well. In addition, McGinnis et al. (2002) have argued that compared with the TDT (when including genotyped parents), case–control samples require fewer individuals to achieve a similar level of power—contrasting this with our TDT design, where parents are absent, it is plausible that the smaller case–control sample was sufficiently powered to detect the association signal. Notwithstanding these possibilities, our results with current smoking need careful replication and currently should be viewed with some caution. A number of emerging genome-wide association studies (GWAS) may provide clues. To date, we are not aware of any GWAS that has implicated polymorphisms in *GABRA2*—however, the candidate genes component of the NICSNP GWAS (Bierut et al., 2007; Saccone et al., 2007) reported evidence for association with *GABRA4* with follow-up analyses also finding considerable support for the role of *GABRA2* (unpublished data).

Given that we found some evidence for association between *GABRA2* and quantitative measures of DSM-IIIR and DSM-IV AD in our sample, we expected to detect significant associations with the diagnostic DSM-IIIR and DSM-IV AD phenotypes. However, only a trend towards association ($p < 0.1$) with DSM-IIIR dependence was observed. The low levels/absence of association for the binary measures of AD may be because of several factors. First, the binary trait (within-family) association analyses excluded both MZ families and families with only 1 twin available. The reduction in the number of informative (phenotyped) individuals included in our analyses resulted in an overall loss in power. Second, our community-based twin sample is not as severely affected by AD as other samples that have shown evidence for association between *GABRA2* and AD, such as the COGA sample selected for multiple affected family members. Therefore, our sample

may have a lower genetic load with respect to AD. Correspondingly, when Dick et al. (2006) compared the onset of AD across the lifespan in a combined sample of COGA families and control families with that of a reduced sample composed of control families alone, they observed less significant allelic effects among the controls ($p = 0.013$ vs. 0.0035). Nevertheless, heritability for AD is high (47 to 75%) and the sibling relative risk is approximately two within our sample (Heath et al., 1997).

The allelic effects which we were able to detect are associated with relative risks of 1.1 to 1.2 for development of AD, particularly dependence with onset of symptoms after the age of 25 (Table 5, Fig. 2). This is consistent with recent estimates for detectable effects of other genes on risk of other diseases (Wellcome Trust Case Control Consortium, 2007). Detection of the expected large number of other polymorphisms producing small but collectively important effects on AD risk will require even larger individual studies and meta-analysis across studies. In this connection, it will also be important to test for heterogeneity across studies and to seek explanations for any heterogeneity in areas such as study characteristics or populations studied. Given that *GABRA2* variation has been shown to affect AD risk in multiple case–control studies based on clinical recruitment, it is notable that only small effects were found in our population-based cohort study. This emphasizes the need to evaluate allelic associations in diverse groups to form a full model of genetic effects on AD and by implication for other complex diseases.

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