Published in final edited form as:
Depress Anxiety. 2009 ; 26(11): 998–1003. doi:10.1002/da.20628.

Association Study between GABA Receptor Genes and Anxiety Spectrum Disorders

Xuan Pham, B.S.[1], **Cuie Sun, B.S.**[1], **Xiangning Chen, Ph.D.**[1], **Edwin J.C.G. van den Oord, Ph.D.**[3], **Michael C. Neale, Ph.D.**[1,2], **Kenneth S. Kendler, M.D.**[1,2], and **John M. Hettema, M.D., Ph.D.**[1]

[1]Department of Psychiatry, Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, VA

[2]Department of Human Genetics, Virginia Institute for Psychiatric and Behavioral Genetics, Virginia Commonwealth University, Richmond, VA

[3]Department of Pharmacy, Center for Biomarker Research and Personalized Medicine, Virginia Commonwealth University, Richmond, VA

Abstract

Background—Human anxiety disorders are complex diseases with relatively unknown etiology. Dysfunction of the GABA system has been implicated in many neuropsychiatric conditions, including anxiety and depressive disorders. In this investigation, we explored four GABA receptor genes for their possible associations with genetic risk for anxiety disorders and depression.

Methods—Our study sample consisted of 589 cases and 539 controls selected from a large population-based twin registry based upon a latent genetic risk factor shared by several anxiety disorders, major depression, and neuroticism. We subjected these to a two-stage protocol, in which all candidate genetic markers were screened for association in stage 1 (N=376), the positive results of which were tested for replication in stage 2 (N=752). We analyzed data from 26 single nucleotide polymorphisms (SNPs) from four GABA receptor genes: *GABRA2*, *GABRA3*, *GABRA6*, and *GABRG2*.

Results—Of the 26 SNPs genotyped in stage 1, we identified two markers in *GABRA3* that met the threshold ($p \leq .1$) to be tested in stage 2. Phenotypic associations of these two markers failed to replicate in stage 2.

Conclusions—These findings suggest that common variation in the *GABRA2*, *GABRA3*, *GABRA6*, and *GABRG2* genes does not play a major role in liability to anxiety spectrum disorders.

Keywords

anxiety disorder; depressive disorder; neuroticism; genetic association study; GABA-A receptors

Introduction

Anxiety is an adaptive response to an impending danger that is integral to an organism's ability to cope with or avoid a threat. Anxiety disorders (ANX) categorize a grouping of syndromes, including generalized anxiety disorder (GAD), panic disorder, and phobias,

Address reprint requests to Dr. Hettema, Department of Psychiatry, Virginia Institute for Psychiatric and Behavioral Genetics, P.O. Box 980126, Richmond, Virginia 23298-0126; jhettema@vcu.edu.

Financial Disclosures: None

where the primary features are abnormal manifestations of anxiety (excessive worry and nervousness, panic attacks, or phobic fears, respectively). These conditions carry a substantial burden of distress and impairment, with lifetime prevalence rates around the world ranging from 5 to 31%, higher than those found for mood or substance use disorders (1). In addition, ANX have high comorbidity rates with each other and with other psychiatric and medical disorders, further compromising the quality of life for sufferers (2).

Much research has been done to understand the genetics of ANX, which carry a heritability of 30% or more (3). Some studies suggest that they share genetic risk factors with depressive disorders and anxious personality traits (4-7). Identifying the specific genes that contribute to the development or maintenance of ANX may offer new insights into the pathophysiology of these disorders and the potential for more effective treatments.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system and regulates many physiological and psychological processes. Both animal and human studies associate variations in GABA in the mammalian forebrain with anxiety and depression (8). For example, the $GABA_A$ receptor is the site of action for anxiolytic drugs of the benzodiazepine and barbiturate classes. Also, GABA concentrations are increased with selective serotonin reuptake inhibitors (SSRIs), the other major drug class currently used to treat ANX as well as depressive disorders. Thus, GABA is strongly implicated in multiple anxiety-related processes.

Although GABA acts through both $GABA_A$ and $GABA_B$ receptors to decrease neurotransmission, we will focus on the $GABA_A$ subtype due to its robustly demonstrated relationship with anxiety (9). In particular, several studies have documented altered benzodiazepine binding at cerebral $GABA_A$ receptors in panic disorder (10;11). Functional $GABA_A$ receptors are typically constructed of five subunits drawn from eight different classes (α, β, etc.) (see (12)for a comprehensive overview). Each subunit is encoded by a distinct gene and differentially expressed in the brain, suggesting that mutation in any one gene could, in principle, contribute to the symptoms of anxiety-related disorders. For the current study, we identified a group of GABA receptor genes with prior suggestive association in anxiety-related phenotypes.

The $GABA_A$ α2 subunit, primarily expressed in the limbic system, likely mediates the anxiolytic effects of benzodiazepines (13). The gene that encodes this protein, *GABRA2*, has been associated with alcohol dependence in several studies (14;15). ANX is known to have high comorbidity rates with alcoholism, and one study suggests that part of the association of this gene with alcohol dependence may be accounted for by anxious temperament (16).

Pharmacological studies have specifically implicated the $GABA_A$ α3 subunit in anxiety (17). Genetic studies of *GABRA3* gene also support its potential role in mood disorder phenotypes. Fiorelli and colleagues (18) provided evidence that mice with global deletion of the *GABRA3* gene had more depression-related behaviors. The human *GABRA3* gene is located in Xq28 region, an area that has been linked to the genetic transmission of bipolar affective disorder (reviewed in (19)). Genetic association has been reported between the *GABRA3* and both bipolar (20) and unipolar (21) mood disorders, although earlier studies failed to detect these associations (22;23).

GABRA6 is involved in several factors contributing to ANX pathology. Sen and colleagues reported an association between a *GABRA6* receptor coding polymorphism and neuroticism (24), a personality trait related to both anxiety and depression. Variation in *GABRA6* has also been associated with increased production of cortisol and increased blood pressure in response to psychological stress (25).

A broad role of *GABRG2* in anxiety-related behavior was demonstrated by Crestani and colleagues, whereby γ2 heterozygous mice with resulting reduced $GABA_A$ receptor clustering showed increased fear of a novel environment (26). These mice also exhibited increased reactivity toward naturally aversive stimuli and enhanced responses to trace fear conditioning. We are aware of no prior association analyses of this gene with human internalizing phenotypes.

Due to the tentative evidence available thus far, we sought to further assess the potential genetic association between these four GABA receptor genes and ANX.

Methods and Materials

Subjects

The subjects in this study are participants in the longitudinal population-based Virginia Adult Twin Study of Psychiatric and Substance Use Disorders (VATSPSUD) (27;28). All subjects were Caucasians born in Virginia. Their age (mean ± SD, range) at time of last interview was (37 ± 9 years, 20-58) for men and (36 ± 8 years, 21-62) for women. Approval of the local institutional review board was obtained from all subjects before data collection.

Diagnostic Measures

We obtained lifetime psychiatric diagnoses via face-to-face or telephone structured psychiatric interview based on the Structured Clinical Interview for DSM-III-R (SCID) (29). We used DSM-III-R (30) diagnostic criteria to assess lifetime major depression, modified DSM-III-R criteria for lifetime generalized anxiety disorder and panic disorder (31;32), and an adaptation of DSM-III criteria for phobias (33). Neuroticism was assessed using the 12 items from the short form of the Eysenck Personality Questionnaire (EPQ) (34) via self-report questionnaire.

Sample Selection

As described previously (35), we used a two-stage association design in which candidate loci were screened in stage 1, the positive results of which were tested for replication in stage 2. The parameters for this design were calculated using the LGA program (36) to achieve 80% power to detect markers that explained 1-2% of the variance of the liability distribution while controlling the false discovery rate at 0.1 (37). Using the extreme selection strategy outlined below, LGA indicated that we needed about 350 subjects in the stage 1 and 1,000 in the stage 2 sample to achieve this. If any of the markers genotyped in stage 1 met the estimated threshold *p*-value of 0.1 or less, they are then followed up in the stage 2 sample.

Starting with a sample of 9270 twin subjects, we used multivariate structural equation modeling to estimate a latent genetic risk factor for neuroticism that is highly correlated (range 0.6-0.8) with genetic susceptibility to major depression, generalized anxiety disorder, panic disorder, agoraphobia, and social phobia (7). One member from each twin pair for whom DNA was available (N=3176 pairs) was selected as a case or control based upon the pair scoring above the 80th or below the 20th percentile, respectively, of the genetic factor extracted from the above analysis. Thus, subjects selected for genotyping (and their co-twins) were determined to be high (cases) or low (controls) on genetic susceptibility for several highly related internalizing phenotypes. This produced a total sample of 1,128 independent subjects for genotyping, consisting of 589 cases (350 males, 239 females) and 539 controls (343 males, 196 females). Using the parameters prescribed by the LGA program, 376 and 752 subjects were used in stages 1 and 2, respectively.

Statistical Analysis

We used Pearson's χ^2 tests to assess allelic or genotypic differences by marker between cases and control subjects, separately by stage to check for consistency of results across the two stages. For analysis of GABRA3 located on the X chromosome, we performed the analyses for men and women separately. We used the program HAPLOVIEW 4.1 (38) to test for Hardy-Weinberg equilibrium (HWE) violations (threshold of p=0.01) and to characterize linkage disequilibrium (LD) between the markers in our sample.

Genotyping

DNA was extracted from buccal epithelial cells obtained via cytology brushes (39). Reactions were performed in 96-well plates SNPs were genotyped by the 5′ nuclease cleavage assay (also called TaqMan method) (40). Reactions were performed in 98-well plates with 5 μl reaction volume containing 0.25 μl of 20× Assays–on-Demand™ SNP assay mix, 2.5 μl of TaqMan universal PCR master mix, and 5 ng of genomic DNA. Each 96-well plate contains samples for either cases or control, and these are intercalated onto a single 384-well plate within a genotyping run to reduce the risk of batch effects differentially affecting cases versus controls. The conditions for PCR were initial denaturizing at 95 °C for 10 minutes, followed by 40 cycles of 92 °C for 15 seconds and 60 °C for 1 minute. After the reaction, fluorescence intensities for reporter 1 (VIC, excitation = 520 ±10 nm, emission = 550 ± 10 nm) and reporter 2 (FAM, excitation = 490 ±10 nm, emission = 510 ± 10 nm) were read by the Analyst fluorescence plate reader (LJL Biosytems, Sunnyvale, CA). Genotypes were scored by a Euclidian clustering algorithm and and checked for deviations from Hardy-Weinberg equilibrium. We performed duplicate genotyping on a subset of plates as a quality control check and for any assays that did not perform optimally.

We selected SNP markers with MAF > 0.05 in each of the four GABA genes with the aim to tag the major haplotypes in the Caucasian panel of the HapMap project (41). Specifically, we used pair-wise tagging in the Tagger module of HAPLOVIEW 4.1 (38) with HapMap Phase II (release 22) data to select SNPs that captured > 80% of the HapMap markers with r2 > 0.8. We note that the decision to capture only 80% of the markers was a tradeoff between competing ideals of minimizing genotyping cost and maximizing coverage of the genes. This provided 3 tagging SNPs for *GABRA2*, 7 SNPs for *GABRA3*, 3 SNPs for *GABRA6*, and 12 SNPs for *GABRG2*.

Results

The genotype and allele frequencies and χ^2 association results for all the markers in the four GABA genes genotyped in stage 1 are listed in tables 1-4. For *GABRA2*, *GABRG2* and *GABRA6*, none of the markers passed the stage 1 p-value threshold of $p < 0.1$. All markers in these three genes were within HWE. In the *GABRA3* gene located on the X chromosome, markers 3 and 4 met the threshold p-value of $p < .1$. (Note: because males are hemizygous for X chromosome markers, HWE and genotypic association testing were performed only for the female subjects.) In *GABRA3*, all markers were in HWE except rs4828694, which was then excluded from further analyses. We genotyped markers 3 and 4 from *GABRA3* gene in the stage 2 sample, but their association failed to replicate (allelic p-values 0.63 and 0.32, respectively).

Discussion

Previous research has implicated the potential involvement of the GABA system in anxiety disorder phenotypes (ANX). In this study, we investigated the possible associations between ANX and four GABA receptor genes, *GABRA2*, *GABRA3*, *GABRA6*, and *GABRG2*. We

analyzed SNPs tagging the major allelic variation in these genes in a sample selected for extremes of common genetic risk across ANX, major depression, and neuroticism. The resulting sample of 589 cases (high genetic risk) and 539 controls (low genetic risk) were entered into a 2-stage association study in which all markers were screened in stage 1, with those showing suggestive association analyzed in stage 2.

In the stage 1 analyses, all markers, with the exception of two in the *GABRA3* gene, failed to meet our criterion of $p < .1$ for further genotyping in stage 2. These two markers, however, did not exhibit association in the larger stage 2 sample. Our 2-stage association design with relatively high detection power revealed no significant association between these four GABA genes and ANX.

The involvement of the GABA system as a whole has been strongly linked to several neuropsychiatric phenotypes, especially ANX. Prior research found preliminary, mostly tentative evidence for association of various GABA receptor genes and a range of anxiety-related phenotypes. If these genes do contain susceptibility loci for ANX, there are a number of possible reasons why we may not have detected a significant association signal. First, our 2-stage study design, while providing a balance between Type 1 and Type 2 errors, may have reduced the overall power to detect variants of small effect size, since we genotype all of the markers in only the smaller stage 1 screening sample. Second, we selected only four of many GABA subunit receptor genes for study, leaving open the possibility that other genes from this group may play a role in ANX. Interestingly, a recent follow-up of a genome-wide association study for bipolar disorder found significant association with five GABA receptor genes, all different than those tested herein (42). Third, we took advantage of the HapMap database to select tagging SNPs across the candidate genes rather than select specific, possibly functional markers as was done in some earlier studies. For example, it is likely that our SNP selection was not able to tag the low frequency exonic SNP in the *GABRA6* gene reported to be associated with neuroticism in one prior study (24) (We could not confirm this *in silico*, since this SNP is not currently in the HapMap database.) As noted earlier, we chose tag SNPs with the aim to capture 80% of the markers available in HapMap, thus excluding a smaller proportion of polymorphisms that might be associated with our phenotypes. Finally, we did not test a range of specific phenotypes as had been examined in prior studies; instead, our strategy was to detect association with a common genetic liability to ANX, major depression, and neuroticism. Thus, we cannot rule out involvement of these genes in specific (rather than common) liability to individual disorders. In the context of these potential limitations, the results obtained from this genetic association study suggest that common polymorphisms in the *GABRA2*, *GABRA3*, *GABRA6*, and *GABRG2* genes are unlikely to play a major role in ANX liability.

Acknowledgments

This work was supported by NIH grant K08 MH-66277 and a Pfizer/SWHR Scholars Award (JMH). We acknowledge the contribution of the Virginia Twin Registry, now part of the Mid-Atlantic Twin Registry (MATR), to ascertainment of subjects for this study. The MATR, directed by Dr. J. Silberg, has received support from the National Institutes of Health, the Carman Trust and the WM Keck, John Templeton and Robert Wood Johnson Foundations.

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Table 1

Stage 1 Association Results (N=188 cases, 188 controls) for *GABRA2* (chromosomal band 4p12, length 140.5 Kb)

Marker	Marker ID	Alleles (major)	Group	Genotypes (%)			Genotypic *p*-Value	Alleles (%)		Allelic *p*-Value
				A1/A1	A1/A2	A2/A2		A1	A2	
1	rs279867	G/T (G)	Cases	27.7	48.6	23.7	0.87	52.0	58.0	0.60
			Controls	25.3	49.4	25.3		50.0	50.0	
2	rs279828	A/C (C)	Cases	26.1	52.2	21.6	0.41	52.2	47.8	0.29
			Controls	24.4	47.8	27.2		48.4	51.6	
3	rs1442062	A/G (A)	Cases	51.1	42.7	6.2	0.53	72.5	27.5	0.77
			Controls	52.0	39.0	9.0		71.5	28.5	

Table 2

Stage 1 Association Results (N=188 cases, 188 controls) for *GABRA3* (chromosomal band Xq28, length 284.2 Kb): males [M] (N=196), females [F] (N=180), and together [All]

Marker	Marker ID	Alleles (major)	Group	Genotypes (%)			Genotypic *p*-Value	Alleles (%)		Allelic *p*-Value		
				A1/A1	A1/A2	A2/A2	F [a]	A1	A2	All	M	F
1	rs11094547	C/T (C)	Cases	63.9	15.8	20.2	0.18	78.0	22.0	0.18	0.15	0.57
			Controls	68.5	19.1	12.4		73.0	27.0			
2	rs12833553	A/G (G)	Cases	79.8	11.8	8.4	0.21	85.2	14.8	0.23	0.61	0.27
			Controls	85.1	7.2	7.7		88.7	11.3			
3	rs6627221	C/T (T)	Cases	72.2	15.0	12.8	0.47	80.4	19.6	0.089*	0.13	0.31
			Controls	80.4	10.9	8.7		85.9	14.1			
4	rs2201169	C/T (T)	Cases	76.9	13.4	9.7	0.38	83.0	17.0	0.088*	0.27	0.19
			Controls	83.8	9.5	6.7		88.1	11.9			
5	rs11797836	A/G (A)	Cases	83.9	11.5	4.6	0.46	88.5	11.5	0.37	0.66	0.20
			Controls	86.8	8.0	5.2		90.9	9.1			
6	rs5969896	A/G (G)	Cases	82.4	11.9	5.7	0.90	88.2	11.8	0.71	0.27	0.70
			Controls	81.2	9.9	8.9		87.2	12.8			

[a] Because GABRA3 is located on the X chromosome, genotypic values are available only for females

* p values that met the stage 1 screening threshold p < .1.

Table 3

Stage 1 Association Results (N=188 cases, 188 controls) for *GABRA6* (chromosomal band 5q34, length 16.9 Kb)

Marker	Marker ID	Alleles (major)	Group	Genotypes (%)			Genotypic *p*-Value	Alleles (%)		Allelic *p*-Value
				A1/A1	A1/A2	A2/A2		A1	A2	
1	rs1992647	C/T (T)	Cases	40.1	45.0	14.9	0.99	62.6	37.4	0.95
			Controls	40.1	44.6	15.4		62.4	37.6	
2	rs13172914	C/T (C)	Cases	33.9	46.1	20.0	0.99	56.9	43.1	0.92
			Controls	33.3	46.5	20.2		56.6	43.4	
3	rs6883758	C/G (C)	Cases	71.2	25.9	2.9	0.90	84.2	15.8	0.65
			Controls	69.0	27.8	3.2		82.9	17.1	

Table 4

Stage 1 Association Results (N=188 cases, 188 controls) for *GABRG2* (chromosomal band 5q34, length 87.9 Kb)

Marker	Marker ID	Alleles (major)	Group	Genotypes (%)			Genotypic *p*-Value	Alleles (%)		Allelic *p*-Value
				A1/A1	A1/A2	A2/A2		A1	A2	
1	rs17060039	C/T (T)	Cases	81.6	18.4	0	0.29	90.8	9.2	0.32
			Controls	85.9	14.1	0		92.9	7.1	
2	rs183294	C/T (C)	Cases	40.7	43.4	15.9	0.47	65.9	34.1	0.31
			Controls	43.4	45.1	11.5		62.4	37.6	
3	rs209353	C/T (T)	Cases	31.2	50.9	17.9	0.85	56.6	43.4	0.87
			Controls	32.0	48.0	20.0		56.0	44.0	
4	rs209358	C/T (T)	Cases	34.1	44.7	21.1	0.76	56.4	43.6	0.79
			Controls	33.3	48.1	18.6		57.4	42.6	
5	rs211037	C/T (C)	Cases	57.2	38.9	3.9	0.28	76.7	24.4	0.73
			Controls	58.5	34.1	7.4		75.6	24.4	
6	rs211032	C/T (C)	Cases	47.2	42.2	10.6	0.46	68.3	31.7	0.28
			Controls	51.1	41.8	7.1		72.0	28.0	
7	rs210984	G/T (T)	Cases	69.5	28.8	1.7	0.86	83.9	16.1	0.94
			Controls	68.6	30.3	1.1		83.7	16.3	
8	rs169793	A/T (A)	Cases	32.9	47.2	19.9	0.29	56.5	43.5	0.11
			Controls	39.9	45.2	14.9		62.5	37.5	
9	rs17060106	A/C (A)	Cases	71.7	26.7	1.6	0.95	85.0	15.0	0.77
			Controls	70.1	28.3	1.6		84.2	15.8	
10	rs12520992	A/C (A)	Cases	73.1	23.6	3.3	0.86	84.9	15.1	0.57
			Controls	75.6	21.6	2.8		86.4	13.6	
11	rs211014	A/C (C)	Cases	59.4	35.0	5.6	0.75	76.9	23.1	0.73
			Controls	59.9	36.3	3.8		78.0	22.0	
12	rs424740	A/T (T)	Cases	36.7	52.8	10.5	0.16	63.1	36.9	0.67
			Controls	39.3	44.4	16.3		61.5	38.5	