Influence of RGS2 on Anxiety-Related Temperament, Personality, and Brain Function

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Context: Although anxiety disorders are heritable, their genetic and phenotypic complexity has made the identification of susceptibility genes difficult. Well-validated animal models and intermediate phenotypes provide crucial tools for genetic dissection of anxiety. The gene encoding regulator of G protein signaling 2 (Rgs2) is a quantitative trait gene that influences mouse anxiety behavior, making its human ortholog (RGS2) a compelling candidate gene for human anxiety phenotypes.

Objective: To examine whether variation in RGS2 is associated with intermediate phenotypes for human anxiety disorders.

Design: Family-based and case-control association analysis of single-nucleotide polymorphisms at the RGS2 locus in 3 independent samples.

Setting: Massachusetts General Hospital, University of California, San Diego, and San Diego State University.

Participants: Study participants included a family-based sample (n=119 families) of children who underwent laboratory-based assessments of temperament (behavioral inhibition), a sample of 744 unrelated adults who completed assessments of extraversion and introversion, and 55 unrelated adults who underwent functional magnetic resonance imaging measures of response to emotional faces.

Main Outcome Measures: Laboratory-based behavioral measures of childhood temperament, self-report measure of personality, and functional magnetic resonance imaging response to emotion processing.

Results: Markers spanning RGS2 were associated with childhood behavioral inhibition, a temperamental precursor of social anxiety disorder (haplotype P=3×10^{-5}; odds ratio, 2.99 in complete trios). In independent samples, RGS2 markers, including rs4606, which has previously been associated with RGS2 expression, were also associated with introversion (a core personality trait in social anxiety disorder) and with increased limbic activation (insula cortex and amygdala) during emotion processing (brain phenotypes correlated with social anxiety). The genotype at rs4606 explained 10% to 15% of the variance in amygdala and insular cortex activation to emotional faces.

Conclusions: These results provide the first evidence that a gene that influences anxiety in mice is associated with intermediate phenotypes for human anxiety disorders across multiple levels of assessment, including childhood temperament, adult personality, and brain function. This translational research suggests that some genetic influences on anxiety are evolutionarily conserved and that pharmacologic modulation of RGS2 function may provide a novel therapeutic approach for anxiety disorders.

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Anxiety disorders are the most common class of psychiatric disorders and are associated with a substantial burden of illness and more than $80 billion in annual costs to society. Anxiety disorders are known to be familial and heritable, but the identification of susceptibility genes has been difficult to accomplish for several reasons. The etiology of these disorders is thought to reflect the effects of multiple genes of individually modest effect interacting with environmental factors. Limited understanding of the underlying neurobiological mechanism means that many genes are plausible risk candidates, but few are compelling. Beyond this genetic and biological complexity, considerable phenotypic complexity exists for anxiety disorders. Although the constellations of symptoms used as diagnostic criteria in the DSM-IV have been useful for clinical practice, it is unlikely that they are the optimal phenotype definitions for genetic analyses. Family, twin, and linkage studies suggest that genes confer susceptibility to anxiety proneness in a manner that cuts across clinical diagnostic labels. Although identifying anxiety susceptibility genes is a formidable challenge, well-validated animal models and intermediate phenotypes, including anxious temperament and functional neuroimaging phenotypes, provide crucial tools. A quantitative trait locus on mouse chromosome 1 has been the most widely

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replicated locus linked to anxious temperament phenotypes in mice. Yalcin et al fine-mapped this locus and identified the gene encoding regulator of G protein signaling 2 (Rgs2) as a quantitative trait gene underlying this linkage signal. Rgs2 knockout mice exhibit increased anxiety and fear behavior, altered hippocampal synaptic plasticity, and elevated sympathetic tone. The Rgs2 protein, which is expressed in cortical and limbic brain regions, is part of a family of proteins that accelerate deactivation of G proteins to reduce G protein–coupled receptor (GPCR) signaling. Neurotransmitters strongly implicated in the biological basis of anxiety, including serotonin and norepinephrine, act at GPCRs. We previously observed modest evidence of linkage between markers that encompass RGS2 and a phenotype of anxiety disorder proneness in a targeted genome screen. Thus, convergent evidence implicates RGS2 as a compelling candidate locus underlying anxiety proneness in humans. We examined whether variation at the RGS2 locus influences intermediate phenotypes for anxiety disorder at the level of behavior and brain function. We first examined the association of RGS2 markers with behavioral inhibition to the unfamiliar (BI), an anxiety-related form of temperament characterized by a tendency to be shy, avoidant, and behaviorally restrained in situations that are novel or unfamiliar. Mouse models of unconditioned and novelty-induced fear responses closely parallel behavioral and biological features of human BI, including inhibition of behavior and increased sympathetic nervous system reactivity. We next examined the BI-related adult personality trait of introversion (low extraversion), which is also characterized by inhibition and avoidant behavior and, finally, limbic responses to emotional faces on functional magnetic resonance imaging (fMRI), a neuroimaging phenotype linked to BI, anxiety proneness, and social anxiety disorder (SAD).

METHODS

SAMPLE

Behavioral Inhibition Sample

Participants were recruited from a sample of families who had participated in a study of children at risk for anxiety disorders conducted at Massachusetts General Hospital. Details of the study sample and behavioral assessments are provided elsewhere. Children from these families were classified as “inhibited” or “not inhibited” on the basis of a behavioral assessment conducted at the ages of 21 months, 4 years, or 6 years. A total of 119 families that included at least 1 child who had undergone behavioral assessments were available at the time of these analyses. The self-reported race of all but 9 families was white. The protocol was approved by the Massachusetts General Hospital institutional review board. After complete description of the study, parents provided written informed consent for themselves and their children, who also provided oral or written assent.

Personality Sample

Participants (n = 744; 516 female) were recruited from among undergraduate psychology students at San Diego State University (SDSU). Participants had blood drawn for genetic studies and completed questionnaires. Participants gave informed written consent to participate in this part of the study, which was approved by the Human Research Protection Programs at both the SDSU and University of California, San Diego. Participants received $25 for providing the blood sample. Power estimates indicate that the sample provides more than 85% power to detect an association at α < .05 with a quantitative trait locus explaining as little as 1.5% of the trait variance.

Neuroimaging Sample

Participants. Initially, approximately 3000 SDSU undergraduate students participated in screening for a behavioral experiment in return for course credits. Of those individuals who participated in the behavioral study, approximately 1 of 3 expressed a willingness to participate in an MRI study; an estimated 1 of 2 provided consent to be contacted for further assessment, and 1 of 2 of these proved eligible. We obtained 2 different samples, which had been collected during another ongoing project. During this time, our imaging studies shifted from a 1.5-T scanner (Siemens, Erlangen, Germany) to a 3.0-T scanner (Signa EXCITE; GE Healthcare, Milwaukee, Wisconsin). Sample 1 (1.5-T scanner) consisted of 29 healthy, right-handed individuals (17 women) with a mean (SD) age of 18.2 (0.62) years and a mean (SD) educational level of 12.6 (0.60) years. Sample 2 (3.0-T scanner) consisted of 26 healthy, right-handed individuals (24 women) with a mean (SD) age of 21.0 (2.6) years and a mean (SD) educational level of 14.5 (1.4) years. All study participants underwent the Structured Clinical Interview for DSM-IV to identify anxiety and mood disorders and were excluded if they were currently seeking, or had ever sought, treatment for their anxiety or mood symptoms. None of the participants had taken any psychotropic medications in the prior 12 months. Participants consumed less than 400 mg of caffeine daily. This study was approved by the SDSU and University of California, San Diego, institutional review boards. All participants gave their informed, written consent to participate and perform the emotion face-processing task during fMRI.

Behavioral Temperament Assessment. As described previously, children underwent laboratory-based temperament assessments at 1 of 3 ages (21 months, 4 years, or 6 years) using age-specific measurement protocols. In brief, the evaluation consisted of behavioral protocols designed to assess the child’s reaction to unfamiliar persons and events during a 90-minute battery. In these protocols, the child, with the mother present, encountered a variety of unfamiliar procedures and tasks, including physiologic measurements and cognitively challenging tasks administered by an unfamiliar female examiner, and their behavioral responses were observed and quantified. The assessments were videotaped and scored by raters who were blind to the assessment of psychopathologic conditions in the children and their parents and blind to genotype status. The relevant dependent variables were behavioral signs of uncertainty, including fretting and crying, cessation of vocalization or activity, retreat or withdrawal from an unfamiliar event, and frequency of smiles and spontaneous comments (see Rosenbaum et al for full description of coded variables). Studies conducted during the past 20 years have established that these variables differentiate inhibited from uninhibited children between the ages of 1 year and 8 years. As in our previous genetic studies of BI, children were classified as inhibited if they met at least 1 of 3 prespecified categorical definitions of BI; children who met none of these definitions were considered unaffected. The genotyped families included 73 children with BI and 89 children without BI (total, 162 children). The sample included 77
two-parent families that comprised 114 trios, 41 single-parent families (including 17 with more than 1 genotyped offspring and 6 with phenotyped sibling pairs), and 1 sibling pair with no parental genotypes.

Self-report Personality Assessment. The NEO-Personality Inventory—Revised is a widely used, 240-item (plus 3 validity items) self-report measure of personality, grouped into 5 major domains: neuroticism (N), extraversion (E), openness to experience (O), conscientiousness (C), and agreeableness (A). Some individuals completed a shorter (60-item) version of the NEO, the NEO-Five Factor Inventory, which provides domain scores (expressed as T scores) that are highly correlated with those obtained from the full instrument. T scores were calculated directly from college-age, sex-specific norms. The phenotype of interest was extraversion T scores.

FUNCTIONAL MRI

Task

During fMRI, each participant was tested with a slightly modified version of the emotion face assessment task (see Hariri et al17). During each 5-second trial, a participant is presented with a target face (on the top of the computer screen) and 2 probe faces (on the bottom of the screen) and is instructed to match the probe with the same emotional expression to the target by pressing the left or right key on a button box. A block consists of 6 consecutive trials during which the target face is angry, fearful, or happy. During the sensorimotor control task, individuals were presented with 5-second trials of either wide or tall ovals or circles in an analogous configuration and instructed to match the shape of the probe to the target. We did not use neutral faces as a comparator because there is mounting evidence that neutral faces are not actually processed as neutral. Each block of faces and of the sensorimotor control task was presented 3 times in a pseudorandomized order. A fixation cross that lasted 8 seconds was interspersed between each block presented at the beginning and end of the task (resulting in 14 fixation periods). For each trial, response accuracy and reaction time data were obtained. There were 18 trials (3 blocks of 6 trials) for each face set and for shapes. The whole task lasted 512 seconds (matching the scan length).

Image Acquisition (1.5 T)

During the task, 1 blood oxygenation level–dependent (BOLD) fMRI run was collected for each study participant using a 1.5-T scanner (Siemens; T2-weighted echo planar imaging; repetition time [TR], 2000 milliseconds; echo time [TE], 40 milliseconds; 64 × 64 matrix; 20 4-mm axial sections; 256 repetitions). During the same experimental session, a T1-weighted MPRAGE image (MPRAGE; TR, 11.4 milliseconds; TE, 4.4 milliseconds; flip angle, 10°; field of view [FOV], 256 × 256; 1-mm3 voxels) was obtained for anatomical reference. For preprocessing, voxel time series were interpolated to correct for nonsimultaneous second acquisition within each volume and corrected for 3-dimensional motion.

Image Acquisition (3 T)

During the task, an fMRI run sensitive to BOLD contrast was collected for each participant using a 3.0-T scanner (Siemens EXCITE) (T2-weighted echo planar imaging; TR, 2000 milliseconds; TE, 32 milliseconds; FOV, 250 × 250 mm2; 64 × 64 matrix; 30 2.6-mm axial sections with a 1.4-mm gap; 256 scans). The fMRI acquisitions were time-locked to the onset of each trial. During the same experimental session, a high-resolution T1-weighted image (spoiled gradient recalled; TI relaxation time, 450; TR, 8 milliseconds; TE, 4 milliseconds; flip angle, 12°; FOV, 356 × 256; 1-mm3 voxels) was obtained for anatomical reference.

Genetic Methods

Selection of Single-Nucleotide Polymorphisms. For the analysis of B1 temperament, single-nucleotide polymorphisms (SNPs) were selected to capture genetic variation across the RGS2 locus. The SNPs were selected from a genomic region that comprised the RGS2 gene, 25 kb of the 5’ flanking sequence, and 10 kb of the 3’ flanking sequence as defined in genome build hg17 using the phase 1 International HapMap Web site (http://www.hapmap.org). Ten tagging SNPs were selected from this region using Tagger (http://www.broad.mit.edu/mpg/tagger/) with r2 > 0.8 and the “aggressive” tagging algorithm. We also included 3 SNPs in the gene (rs2746071, rs2746073, rs17697363, rs4606, and rs3767488) that were not available in HapMap at the time of selection but were found in dbSNP (http://www.ncbi.nlm.nih.gov/SNP). Linkage disequilibrium relationships within the SNP set were calculated using the Gabriel criteria as implemented in Haplovie (http://www.broad.mit.edu/mpg/haplovie/index.php). A single haplotype block encompassed the gene and all markers with the exception of the 5’ marker rs3856223, which nevertheless was in strong linkage disequilibrium with markers in the block and was thus included in the haplotype analysis. The final set of 15 SNPs achieved an average density of 1 SNP per 2 kb across a 32-kb region and an average r2 of 0.90 with untyped HapMap markers that have minor allele frequency of 10% or more, indicating excellent coverage of variation across the locus. Because our marker set included 5 SNPs not included in HapMap, this average r2 is an underestimate of the true variation captured by our set. For the NEO-E analyses, we selected a subset of the 15 markers based on the results of the BI analysis, and for the fMRI analyses, we selected rs4606 because of the recent report of its functional significance.

Genotyping Method. Genotyping of SNPs in the BI family sample was performed by mass spectrometry (Sequenom, San Diego, California). Markers were retained for analysis if they met the following criteria: (1) no significant deviation from Hardy–Weinberg equilibrium (P > .01) and (2) minimum call rate of 85% (average call rate, 96.4%). For 1 SNP (rs4606), genotypes were repeated and the resulting genotypes combined (overall call rate, 98%). All 15 markers were thus retained. Affected and unaffected individuals were spread across genotyping plates to avoid bias due to plate-specific genotyping error.

Genotyping for the NEO-E and fMRI Samples. The SNPs were genotyped with a fluorogenic 5’ nucleotide assay method (ie, the TaqMan technique) using the ABI PRISM 7900 Sequence Detection System (ABI, Foster City, California). All genotypes were assayed in duplicate, and discordant genotypes (which ranged from 0% to 3.3%, depending on the marker) were discarded. All markers were in Hardy–Weinberg equilibrium.

Ancestral Proportion Scores. The ancestries of the study participants were estimated using a set of unlinked genetic markers by Bayesian cluster analysis, using STRUCTURE software (http://pritch.bsd.uchicago.edu/software.html). The markers were the set of short tandem repeats selected for ancestry information and described previously. STRUCTURE implements Bayesian cluster modeling that can infer population genetic patterns without prior information of population origins.
The model was specified as “admixture” and “allele frequencies correlated,” with 100 000 burn-in and 100 000 Markov chain Monte Carlo iterations. Analysis with STRUCTURE indicated adequate fit for a 3-class solution, which was used in the NEO-E and fMRI analyses.

STATISTICAL ANALYSES

Family-Based Association Analysis of Temperament

Family-based association analyses of the BI sample were performed using the Family Based Association Test (FBAT) Program 1.7.1 (http://www.biosstat.harvard.edu/~fbat/default.html). The offset option of FBAT (using an offset equal to the sample prevalence) was used to incorporate all offspring who were phenotyped and genotyped. Haplotype-specific and global haplotype tests were performed using permutation (N=100 000 cycles) by the hbat-p option. The min-p test was used to calculate a global haplotype test. This test evaluates the statistical significance of the smallest observed P value among all the individual haplotypes and estimates a P value by permutation. The odds ratio associated with the risk haplotype was calculated from complete trios using WHAP (http://pngu.mgh.harvard.edu/purcell/whap/).

NEO-E (Introversion) Association

Single-marker and haplotype-based analyses of the NEO-E quantitative trait were performed using WHAP, with empirical P values determined by permutation testing. Sample mean and variance were fixed to optimize model stability in single-marker and haplotype analyses. Analyses incorporated a 3-class population solution derived from STRUCTURE to avoid confounding due to population stratification.

Our analytic strategy was based on sequentially maximizing the prior probability of association and reducing multiple testing by first testing the full set of markers for the BI phenotype, retaining SNPs with the strongest signals for the introversion analysis, and then focusing on the most relevant markers for the fMRI analysis. The fMRI analysis focused on rs4606 in particular because it is the marker that has been associated with gene expression levels. To further support these results and confirm they were not a function of genotyping error, we also examined rs10801152 (the SNP that showed the strongest statistical evidence across the BI and introversion analyses).

fMRI Analysis

All structural and functional image processing was performed with the Analysis of Functional Neuroimages (AFNI) software package.19 Echoplanar intensity images were coregistered to the 128th image using a 3-dimensional coregistration algorithm. The time series of the alignments in the x, y, z, and roll, pitch, yaw direction was used to obtain motion regressors for each study participant. Because small motion corrections are similar in angle (eg, roll) and displacement (eg, x), we used only 3 motion parameters (roll, pitch, yaw) as nuisance regressors to account for motion artifacts. The 4 orthogonal regressors of interest were (1) happy, (2) angry, (3) fearful, and (4) circle or oval (ie, shape) sensorimotor condition. These regressors were convolved with a modified y-variate function to account for the delay and the dispersion brain response of the BOLD-fMRI signal due to hemodynamics.22 Additional regressors were used to model residual motion in the roll, pitch, and yaw directions and baseline and linear trends. The AFNI program 3dDeconvolve was used to calculate the estimated voxel-wise response amplitude. A gaussian filter with a full width at half maximum of 4 mm was applied to the voxelwise percentage of signal change data to account for individual variations in the anatomical landmarks.

Data from each participant were normalized to Talairach coordinates. Whole-brain analyses were followed by a priori analysis of regions of interest using masks (defined by the Talairach demon atlas)24 in the bilateral amygdala, insular cortices, ventromedial prefrontal cortex, and primary visual cortex. On the basis of these areas of interest, it was determined via simulations that a voxel-wise a priori probability of .05 would result in a corrected clusterwise activation probability of .05 if a minimum volume of 128 µL and 2 connected voxels (in the amygdala, which is a small structure) or 512 µL and 8 connected voxels (in all other regions of interest) was considered. The areas of interest were superimposed on each individual’s voxelwise percentage of signal change brain image. Only activations within the areas of interest, which also satisfied the volume and voxel connection criteria, were extracted and used for further analysis. The corrected voxelwise probabilities are as follows: amygdala, P<.012; insular cortex, P<.000069; medial prefrontal cortex, P<.00014; and visual cortex, P<.000070. These corrected voxel probabilities are based on Monte Carlo simulations using AFNI’s program AlphaSim using the filtered data and the a priori defined regions of interest.

To maximize power by increasing the number of individuals homozygous for the putative rs4606 risk (G) allele, we pooled the 1.5-T and 3-T data sets rather than considering them separately. Specifically, for these analyses we used a regression approach with a case-control design (1.5 T or 3 T) and ancestral informative markers (AIM 1 and AIM 2) coefficients as covariates and genotype for rs4606 (CC=0, CG=1, GG=2) as the variable of interest. Associations with a significant gene effect (ie, voxelwise partial correlation coefficient with P<.05) that also fulfilled the volume-threshold cluster condition within the regions of interest were extracted, and additional statistical analyses were conducted using SPSS statistical software, version 15.0 (SPSS Inc, Chicago, Illinois). Results of the regression analyses are presented in eTable 1 (available at http://www.archgenpsychiatry.com).

ASSOCIATION OF RGS2 WITH CHILDHOOD ANXIOUS TEMPERAMENT

We first examined whether RGS2 influences behavioral and neurobiological phenotypes underlying human anxiety by examining a form of human anxious temperament that shares core phenotypic and biologic features with mouse models of unconditioned and novelty-induced fear behavior. Behavioral inhibition to the unfamiliar is a heritable temperament profile characterized by a tendency to be shy, avoidant, and behaviorally restrained in situations that are novel or unfamiliar.23 Biological features of BI include evidence of increased sympathetic tone and limbic hyperreactivity to novel stimuli.9,25 In addition, BI is a familial and developmental risk factor for anxiety disorders (and, in particular, SAD)20 but has greater estimated heritability than the diagnostic categories.27 The sample comprised 119 families in which children underwent standardized laboratory-based behavioral assessments of BI, as previously described.13 To capture genetic variation across the RGS2 locus, we genotyped a set of 15 SNPs with an average density of 1 SNP per 2 kb across a 32-kb region spanning

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RGS2 (Figure 1). As indicated in Table 1, Table 2, and Figure 2, 9 of the 15 SNPs tested were associated with BI, including the G allele of the 3' UTR SNP rs4606 (P = .0026), which has been shown to be associated with reduced RGS2 expression in vitro.19 A haplotype that comprised all 15 markers was also associated with BI (permuted P = 3.0 × 10⁻⁵). The odds ratio for BI associated with the risk haplotype, calculated from complete family trios, was 2.99 (95% confidence interval, 1.31-6.84).

ASSOCIATION OF RGS2 WITH SOCIAL ANXIETY-RELATED PERSONALITY IN ADULTS

In adults, social inhibition can be indexed by the personality trait of introversion (low extraversion), which, like BI, is a heritable trait28 characterized by low levels of sociability and aversion to large groups. Longitudinal data suggest that childhood BI is a developmental precursor of introversion (but not neuroticism).29 Like BI, introversion is associated with risk for anxiety disorders, including SAD.30 If variants in RGS2 are associated with temperamental shyness, we hypothesized that these variants would also be associated with introversion (low extraversion). We genotyped the 4 markers that showed the strongest signal in the BI sample in an independent sample of 744 college undergraduates (228 men and 516 women) who completed the NEO-Personality Inventory–Revised, from which the extraversion scale (NEO-E) can be derived.15 Consistent with our prediction, we observed an association between NEO-E and the same alleles of these 4 markers that were associated with BI (Table 3). A haplotype of these 4 alleles was also associated with

![Figure 1. Schematic of the gene encoding regulator of G protein signaling 2 (RGS2) locus, depicting positions of genotyped single-nucleotide polymorphisms. The lower portion of the figure shows a magnification of the RGS2 locus. Kb indicates kilobase.](image-url)

**Table 1. Association of Behavioral Inhibition to the Unfamiliar With Markers Spanning the RGS2 Locus: Single Marker Results**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Position (hg17)</th>
<th>Major/Minor Alleles</th>
<th>MAF</th>
<th>z (Allele)²</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3856223</td>
<td>189484723</td>
<td>C/T</td>
<td>0.35</td>
<td>1.90 (T)</td>
<td>.057</td>
</tr>
<tr>
<td>rs6670601</td>
<td>189490272</td>
<td>A/C</td>
<td>0.47</td>
<td>0.54 (C)</td>
<td>.59</td>
</tr>
<tr>
<td>rs6670801</td>
<td>189490498</td>
<td>A/G</td>
<td>0.36</td>
<td>1.51 (G)</td>
<td>.13</td>
</tr>
<tr>
<td>rs10801152</td>
<td>189492961</td>
<td>A/T</td>
<td>0.31</td>
<td>2.99 (T)</td>
<td>.0028²</td>
</tr>
<tr>
<td>rs10921267</td>
<td>189492428</td>
<td>G/T</td>
<td>0.27</td>
<td>2.43 (T)</td>
<td>.015</td>
</tr>
<tr>
<td>rs6428136</td>
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<td>T/G</td>
<td>0.27</td>
<td>2.92 (G)</td>
<td>.0036</td>
</tr>
<tr>
<td>rs7531013</td>
<td>189497600</td>
<td>G/A</td>
<td>0.47</td>
<td>0.26 (G)</td>
<td>.79</td>
</tr>
<tr>
<td>rs1342809</td>
<td>189502209</td>
<td>G/T</td>
<td>0.17</td>
<td>2.05 (G)</td>
<td>.040</td>
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<tr>
<td>rs1890397</td>
<td>189502590</td>
<td>G/A</td>
<td>0.49</td>
<td>0.04 (A)</td>
<td>.97</td>
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<td>rs2746071</td>
<td>189509221</td>
<td>A/G</td>
<td>0.29</td>
<td>1.98 (G)</td>
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<tr>
<td>rs2746073</td>
<td>189510884</td>
<td>T/A</td>
<td>0.25</td>
<td>2.57 (A)</td>
<td>.010</td>
</tr>
<tr>
<td>rs17647363</td>
<td>189512155</td>
<td>A/G</td>
<td>0.16</td>
<td>1.53 (A)</td>
<td>.13</td>
</tr>
<tr>
<td>rs4606</td>
<td>189512829</td>
<td>G/G</td>
<td>0.27</td>
<td>3.01 (G)</td>
<td>.0026²</td>
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<tr>
<td>rs3767488</td>
<td>189513296</td>
<td>A/G</td>
<td>0.25</td>
<td>2.78 (G)</td>
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<tr>
<td>rs1819741</td>
<td>189516495</td>
<td>T/C</td>
<td>0.26</td>
<td>3.07 (C)</td>
<td>.0021²</td>
</tr>
</tbody>
</table>

Abbreviations: MAF, minor allele frequency; RGS2, gene encoding regulator of G protein signaling 2.

a z Statistic and overtransmitted allele.

b P values are significant after Bonferroni correction for 15 single-marker tests.
introduction (global and haplotype-specific \( P = .04\)). Although our primary hypothesis was that \( RGS2 \) would be associated with introversion, we performed secondary analyses to determine whether the effect was specific to this trait. In those analyses, we observed no association between \( RGS2 \) markers and the other NEO subscales (neuroticism, openness, conscientiousness, or agreeableness).

ASSOCIATION OF \( RGS2 \) WITH SOCIAL ANXIETY–RELATED BRAIN FUNCTION

In light of previous studies that suggest that BI and social anxiety are mediated by hyperreactivity of brain structures (especially amygdala and insular cortices) thought to underlie anxiety proneness,\(^9\)–\(^12\) we hypothesized that \( RGS2 \) variants associated with BI and introversion would also show association with functional reactivity of these structures during emotion processing. To investigate this, we examined genotype effects on limbic brain reactivity to emotional faces, a neuroimaging assay of anxious temperament. Previous fMRI studies\(^31\) have shown that limbic brain circuits involved in anxiety are activated when individuals view novel or emotional faces. In particular, increased amygdala activation to novel or emotional faces has been associated with inhibited temperament,\(^9\) social anxiety traits,\(^32\) and SAD,\(^12\) although other areas, including the anterior cingulate cortex and the insular cortex, have also been implicated.\(^31,33\) By directly indexing brain function, anxiety-related fMRI phenotypes may provide more proximal and therefore more powerful measures of gene action.

For these analyses, we selected the \( 3' \) UTR SNP rs4606, which has been associated with variation in \( RGS2 \) messenger RNA expression.\(^9\) We genotyped rs4606 in 2 independent groups (29 tested in a 1.5-T magnet and 26 tested in a 3-T magnet) of healthy volunteers drawn from an ongoing study with college-age individuals. To maximize power for analyses of the putative risk allele (rs4606-G), we pooled the samples and included a covariate for the magnets. Individuals in both magnets were tested during fMRI using the same version of a slightly modified emotion face assessment task that has been shown to be sensitive to genetic influence.\(^17,34\) For each 5-second trial, an individual is presented with a target face (on the top of the computer screen) and 2 probe faces (on the bottom of the screen) and is instructed to match the probe with the same emotional expression (happy, sad, or angry) to the target by pressing the left or right key on a button box. During the sensorimotor control task, study participants were presented with 5-second trials of either wide or tall ovals or circles in an analogous configuration and instructed to match the shape of the probe to the target. Several investigators have used this task to show significant activations in the amygdala during the presentation of faces vs the sensorimotor control condition.\(^17,34\) Moreover, we have found previously that the degree of insular cortex activation during this task was modulated by both short-term administration of an anxiolytic and by the degree of anxiety proneness.\(^10,16\) The rs4606 G allele, which showed association with BI and introversion in the analyses described herein, was significantly associated with the degree of left amygdala and bilateral insular cortex activation (Figure 3, Figure 4, and eTable 1). Specifically, in models that control for magnet (1.5 T vs 3 T) and ancestry-informative marker clusters,\(^25\) the rs4606 G allele was independently associated with the extracted average activation.

### Table 2. Association of Behavioral Inhibition to the Unfamiliar With Markers Spanning the \( RGS2 \) Locus: Haplotype Results (>5% Frequency) With Permuted \( P \) Values

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Frequency</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-C-G-T-G-G-G-G-G-G-G-G-G-G-G-C</td>
<td>0.18</td>
<td>( 2 \times 10^{-4} )</td>
</tr>
<tr>
<td>C-C-A-A-C-T-G-G-G-G-G-G-G-G-G-G-G-C</td>
<td>0.08</td>
<td>.75</td>
</tr>
</tbody>
</table>

Global minimal \( P \) permutation test \( 3 \times 10^{-4} \)

Abbreviation: \( RGS2 \), gene encoding regulator of G protein signaling 2.
Table 3. Single Marker and Haplotype Association of 4 RGS2 Markers With Introversion

<table>
<thead>
<tr>
<th>Marker</th>
<th>Risk Allele</th>
<th>LRT</th>
<th>P Value</th>
</tr>
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<tbody>
<tr>
<td>rs10801152</td>
<td>T</td>
<td>7.18</td>
<td>.0074</td>
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<tr>
<td>rs6428136</td>
<td>G</td>
<td>5.54</td>
<td>.019</td>
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<tr>
<td>rs4606</td>
<td>G</td>
<td>4.11</td>
<td>.043</td>
</tr>
<tr>
<td>rs1819741</td>
<td>C</td>
<td>3.81</td>
<td>.051</td>
</tr>
<tr>
<td>4-Marker haplotype</td>
<td>T-G-G-C</td>
<td>4.32</td>
<td>.038</td>
</tr>
</tbody>
</table>

Abbreviations: LRT, likelihood ratio test statistic (1 df); RGS2, gene encoding regulator of G protein signaling 2.

ªAllele associated with introversion.

ªHaplotype-specific test with a minimum haplotype frequency of 5%. Analysis of ancestry-informative markers indicated no effect of population stratification.

On the basis of consistent results derived from a set of different but interrelated anxiety paradigms in independent samples, we observed compelling evidence that RGS2, the ortholog of a mouse anxiety quantitative trait gene, is also associated with anxiety-related phenotypes in humans. A particularly strong effect was seen for childhood BI, which closely parallels behavioral and biologic features of mouse phenotypes influenced by murine Rgs2. Our findings are the first, to our knowledge, to document association of a specific gene with social anxiety across 3 levels of phenotypic analysis: a laboratory-based behavioral measure of childhood temperament, a self-report measure of adult personality, and a neuroimaging measure of functional brain activity.

The finding of RGS2-related activation in the amygdala is analogous to similar findings with the serotonin transporter promoter polymorphism37 and the catechol O-methyltransferase val-met variant.34 In addition, our results are the first, to our knowledge, to demonstrate an association between the insular cortex, a limbic brain region involved in emotional processing,33 and a gene implicated in anxiety. The insular cortex is part of a neural system involved in homeostatic processing of autonomic arousal and visceral changes, signaling executive areas to initiate avoidant behavior and altering self-awareness.33 The insular cortex, medial prefrontal cortex, and amygdala play crucial roles in linking internal physiologic states to external cues or events. Although some investigators have proposed that the connectivity between the amygdala and the medial prefrontal cortex or anterior cingulate is a critical genetically determined factor, dysfunction of which predisposes individuals to anxiety or depression,35 the limited number of high-risk allele individuals in our sample prevented a rigorous test of this hypothesis using functional connectivity measures. Clearly, future investigation will need to examine genetic determinants of functional connectivity between amygdala or insular cortex and other areas that are important for emotion regulation.36

RGS2 is one of a family of regulators of G protein signaling that function as guanosine triphosphatase (GTPase) accelerating proteins, terminating G protein signaling by binding to activated Gα subunits and accelerating the rate of guanosine triphosphate (GTP) hydrolysis. RGS2 regulates G;

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thought to underlie anxiety, including the hippocampus, amygdala, cerebral cortex, hypothalamus, and dorsal raphe nuclei. Neurotransmitters implicated in anxiety, including serotonin, norepinephrine, and dopamine, act at GPCRs. RGS2 has been shown to markedly decrease Gq/H1 signaling by serotonin 2A receptors, which, in turn, play a key role in anxiety and stress responses, as well as response to serotonergic antidepressants. RGS2 has also been shown to regulate hippocampal synaptic plasticity by increasing neurotransmitter release via pre-synaptic Gi/o-mediated Ca2+/H1 channel inhibition. Neuronal RGS2 transcription is modulated by plasticity-inducing synaptic stimuli and by agents known to affect anxiety and mood symptoms, and RGS2 expression has been implicated in experience-dependent development of neural circuits. RGS2-deficient mice exhibit increased anxiety behavior, increased sympathetic tone, altered blood pressure response to a novel environment, and increased urinary norepinephrine excretion—features also reported in human BI. Our results suggest that at least some genetic influences on fear responses to novelty are evolutionarily conserved. The identity of the specific phenotype-influencing variant(s) mediating RGS2 effects on human anxiety phenotypes cannot be determined from these data, although the dense map of SNPs examined in the analysis of BI captures a minimum of 90% of the genetic variation in the region and is likely to have directly or indirectly assayed the relevant variants. Resequencing of the gene in previous studies has not revealed common coding sequence variants. However, the G allele of rs4606, which was associated with anxiety phenotypes in our study, has been associated with reduced RGS2 expression in both peripheral blood mononuclear cells and fibroblasts in hypertensive patients. Reduced RGS2 expression is expected to be associated with anxiety given that deletion of the gene is associated with anxious temperament in mice.

Taken together, our results suggest a model in which genetic variation associated with reduced expression of RGS2 contributes to increased reactivity of limbic brain structures modulating anxious temperament and social anxiety. At a behavioral level, this genetic effect is most evident in direct measurements of inhibited temperament (which itself has been shown to be associated with amygdala reactivity in previous research), with a weaker effect detectable on adult social anxiety–related personality. This model rests in part on the premise that our measures of temperament, personality, and brain function are phenotypically convergent. One way to verify this would be to measure all 3 phenotypes in the same individuals and examine their relationship; this was not possible because BI is based on laboratory-based behavioral temperament observations in young children (who could

Figure 4. Activation differences associated with the rs4606 genotype in bilateral insular cortices for the combined 1.5-T and 3-T samples. The marginal means are obtained relative to the covaried ancestral proportion weights and field strength indicators. For the statistical effect, see eTable 1 (available at http://www.archgenpsychiatry.com). A and B, The bar graphs show the larger of the 2 areas within the right anterior insular cortex (A) and the left anterior insular cortex (B). Error bars indicate SD. C and D, Axial sections in Talairach coordinates at z=0 and z=4 show 2 regions on the left (D) and 1 on the right (C) (in red).
not complete the NEO or undergo functional imaging). However, substantial evidence indicates that BI, introversion, and limbic reactivity are, in fact, convergent phenotypes: prior studies have demonstrated an association between BI measured and introversion, between BI and limbic reactivity to emotional faces, and among all 3 traits (BI, introversion, limbic reactivity to emotional faces) and SAD.11,12,30,46-51

In secondary analyses that examined the specificity of RGS2 effects on personality, we did not observe association with neuroticism, which has been reported to be linked to the 1q region syntenic with mouse chromo-

some 1.52 Our data do not support the hypothesis that RGS2 underlies this linkage signal because our adult sample was powered to detect loci explaining as little as 1.5% of the variance in neuroticism. However, the linked region contains many genes, and it may be that 1 or more of these genes contribute to neuroticism. Of note, however, a recent whole genome association study of neuroticism failed to detect any loci at this region.53 To our knowledge, no linkage or association studies that include loci on 1q have examined the phenotype of introversion. Prior studies suggest that introversion is more specifically related to BI and social anxiety, whereas neuroticism appears to be a nonspecific risk factor for depression and anxiety disorders (especially generalized anxiety disorder).56-57 Although neuroticism mainly captures negative emotionality and worry, introversion is more directly related to social inhibition and shyness, core features of BI. Caspi et al29 examined temperament in 3-year-old children and followed up these children to adulthood. Inhibited temperament at the age of 3 years was associated with introversion at the age of 26 years but was unrelated to adult neuroticism. Gladstone and Parker44 found that an adult measure of BI was strongly and similarly correlated with introversion \((r = 0.75)\) and social anxiety \((r = 0.77)\). In a subsequent study,57 retrospective childhood BI was significantly associated with social phobia but not panic disorder, generalized anxiety disorder, or agoraphobia. Numerous other studies, including longitudinal studies, have confirmed the specific relationship between BI and social anxiety, and we have previously shown in a college sample that introversion is highly and significantly correlated with measures of shyness and social anxiety.38

Further studies will be needed to determine which, if any, anxiety disorder phenotypes are most tightly related to RGS2. Given the association with BI and introversion, 2 traits that are risk factors for SAD, we would predict that SAD is the most likely anxiety disorder to be associated with RGS2. A recent study by Leygraf et al59 reported nominally significant evidence of association between RGS2 markers and panic disorder, although these results would not survive correction for multiple testing. To the extent that genetic effects on DSM-IV anxiety disorders may be smaller than effects on the intermediate phenotypes examined herein, much larger samples may be needed for studies of the clinical disorders.

In conjunction with studies in mouse models, our findings suggest that RGS2 modulators could provide a novel therapeutic approach for the treatment of anxiety disorders. For example, agents that facilitate RGS2 would be expected to inhibit GPCR signaling in response to neurotransmitters targeted by antidepressants that effectively treat anxiety disorders. The hypothesis that anxiety proneness is related to reduced RGS2 expression implies that agents that enhance RGS2 activity would be anxiolytic. The regional expression of RGS2 in limbic and paralimbic brain areas coupled with its selectivity for Gqα-mediated and Gi/o-mediated signaling might enhance the therapeutic action of GPCR-based treatments of anxiety and mood disorders.6

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Author Contributions: Drs Gelernter and Stein contributed equally to this work.

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Additional Information: The e-Tables and e-Figure are available at http://www.archgenpsychiatry.com.

Additional Contributions: The following assisted in this work: Pamela Sklar, MD, PhD, Lisa Watras, BA, Brian Galloway, BA, Priya Moorjani, MS, Alan Simmons, PhD, Jerome Kagan, PhD, and Nancy Snidman, PhD. We are grateful to the individuals and families who participated in this work.

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eFigure. Results of analysis of covariance with magnet and rs4606 genotype as the main variables and ancestral-informative markers 1 and 2 as covariates. All values for the bar graphs are corrected for the covariates. The risk allele rs4606-G was associated with increased activation during the faces relative to the sensorimotor control condition in all areas identified in the pooled magnet analyses described in the text. Error bars indicate SD.
### eTable 1. Multiple Regression Analyses of the rs4606 Genotype for the Constrained Regions of Interest in the Amygdala and Left and Right Insular Cortex

<table>
<thead>
<tr>
<th>Region</th>
<th>Tesla</th>
<th>AIM 1</th>
<th>AIM 2</th>
<th>rs4606</th>
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<td>Left insula (1)</td>
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<td>Coefficient</td>
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<td>P value</td>
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<td>.009</td>
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<td></td>
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<tr>
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<td>.03</td>
<td>.03</td>
<td>.02</td>
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*Tesla indicates the 1.5-T vs the 3-T magnet as a covariate, and AIM 1 and AIM 2 refer to ancestry-informative marker covariates derived from STRUCTURE ([http://pritch.bsd.uchicago.edu/software.html](http://pritch.bsd.uchicago.edu/software.html)). As indicated, the association with the rs4606 genotype explains a significant proportion of the variance in activation, even after adjusting for these covariates. b There were 2 areas of significant activation within the left insula.

### e-Table 2. Multiple Regression Analyses of the rs10801152 Genotype for the Constrained Regions of Interest in the Amygdala and the Left and Right Insular Cortex Defined by the rs4606 Results

<table>
<thead>
<tr>
<th>Region</th>
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<th>AIM 1</th>
<th>AIM 2</th>
<th>rs10801152</th>
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<td>Left insula (1)</td>
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</tr>
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<td>P value</td>
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*The rs10801152 T allele (which was also associated with behavioral inhibition to the unfamiliar and introversion) was associated with left insula and amygdala activation. Tesla indicates the 1.5-T vs the 3-T magnet as a covariate, and AIM 1 and AIM 2 refer to ancestry-informative marker covariates derived from STRUCTURE ([http://pritch.bsd.uchicago.edu/software.html](http://pritch.bsd.uchicago.edu/software.html)). b There were 2 areas of significant activation within the left insula.