Archival Report

Catechol-O-Methyltransferase Contributes to Genetic Susceptibility Shared Among Anxiety Spectrum Phenotypes

John M. Hettema, Seon-Sook An, Jozsef Bukszar, Edwin J.C.G. van den Oord, Michael C. Neale, Kenneth S. Kendler, and Xiangning Chen

Background: Catechol-O-methyltransferase (COMT) has been investigated for its possible role in a wide range of psychiatric phenotypes. In particular, several studies support association of this gene with panic disorder and other anxiety-related traits.

Methods: We examined the COMT gene for association with genetic risk across a range of anxiety spectrum phenotypes. We used multivariate structural equation modeling to select twin pairs scoring at the extremes of a latent genetic risk factor shared by neuroticism, several anxiety disorders, and major depression from a large population-based twin sample. With one member from each of these pairs, the resulting sample of 589 cases and 539 control subjects were entered into a two-stage association study in which genetic markers were screened in stage 1, the positive results of which were tested for replication in stage 2.

Results: The functional val158met polymorphism (rs4680) plus nine other single nucleotide polymorphism markers selected to capture the major allelic variation across the COMT locus were analyzed for differences between cases and control subjects. Although the val (G) allele of rs4680 showed marginally significant association in our combined stage 1 plus stage 2 sample, a high-risk haplotype of this allele with the A allele of rs165599 was significantly over-represented in cases (p = 1.97e-5, odds ratio = 1.95). This haplotype also predicted individual differences in neuroticism and risk for several anxiety disorders and major depression. Consistent with prior studies, our findings are female-specific.

Conclusions: Variations in the COMT gene contribute to genetic risk shared across a range of anxiety-related phenotypes.

Key Words: Anxiety, association study, catechol-O-methyltransferase, depression, genetics, personality

Catechol-O-methyltransferase (COMT), the enzyme involved in the inactivation of catecholamines, has been investigated for its potential role in a broad range of psychiatric phenotypes (1). This gene possesses a common G>A functional polymorphism identified at codon 158 that produces an amino acid change from valine to methionine (2), commonly known as val158met (dbSNP rs4680). Accompanying this is a significant difference in the level of COMT enzymatic activity, with the val allele (G) possessing the higher activity.

The COMT gene has been implicated in pathological anxiety states from animal and clinical studies in a sexually dimorphic manner. Female mice in which COMT was disrupted exhibited changes in anxiety-related behaviors, whereas their male counterparts were affected in measures of aggression (3). Table 1 summarizes available studies of the COMT val158met polymorphism in relation to human anxiety-related symptoms or personality traits. The overall findings suggest a marginal and somewhat inconsistent association between the met allele or met-met genotype and higher levels of anxiety-related measures in females. Two exceptions are the McGrath et al. study (4) and the Kim et al. study (5), both of which reported higher anxiety-related scores in association with the val allele in females. In the only study to examine sources of variation in the COMT locus other than val158met for anxiety-related traits, Stein et al. studied three single nucleotide polymorphisms (SNPs) (including rs4680) that define a haplotype associated with variation in brain expression of COMT (6) in relation to Neuroticism Extraversion Openness (NEO) personality traits neuroticism and extroversion (7). They found lower extroversion and higher neuroticism in females with the met allele, with this relationship becoming more highly significant in haplotypic analyses with combinations of the three SNPs. Prior studies in schizophrenia have also reported multi-marker haplotypes that showed greater association with the phenotype than the val158met polymorphism alone (1,8).

The COMT val158met polymorphism has been tested for association with several anxiety disorders, with panic disorder demonstrating the most consistent findings. The group at Columbia University analyzed COMT for linkage and association with panic disorder in their family-based Caucasian sample of 70 panic disorder pedigrees and 83 parent-offspring triads (9). They found significant linkage for several polymorphisms, including val158met, as well as significant association for several haplotypes made up of combinations of these polymorphisms, finding the high-activity val allele associated with panic disorder. A recent review and meta-analysis of six case-control studies of val158met in relation to panic disorder reported an overall significant association of the val allele with panic disorder in Caucasian samples but a trend toward association of the met allele in Asian samples; their sub-analysis by gender suggests that the association is limited to female subjects (10).

Among studies that have examined COMT in relation to mood disorders, although two small Caucasian case-control studies found no consistent evidence of association between COMT and unipolar depression (11,12), a large European multi-center study...
reported significant association of the val allele with early-onset major depression (13). COMT was also found to predict onset of depressive episodes after exposure to stressful life events in another large European sample (14).

Given the potential role of COMT in a broad range of internalizing phenotypes, in this study we sought to assess the potential association between COMT gene variants and shared genetic risk across a range of anxiety-related phenotypes in a large population-based sample. Specifically, we tested the val158met polymorphism as well as other markers that characterize the major allelic variation around the COMT locus, together with relevant haplotypes. Also, owing to the substantial proportion of reports that were specific to women, we also analyzed our results by gender.

### Methods and Materials

#### Subjects

The subjects in this study derive from the longitudinal population-based VATSPSUD (Virginia Adult Twin Study of Psychiatric and Substance Use Disorders) (15,16). All subjects were Caucasian and 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 before the study, and informed consent 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) (17). We used DSM-III-R (18) diagnostic criteria to assess lifetime major depression, modified DSM-III-R criteria for lifetime generalized anxiety disorder and panic disorder (19,20), and an adaptation of DSM-III criteria for phobias (21) (22). We included agoraphobia and social phobia in the phenotypic modeling used for this study (see following text). Neuroticism was assessed with the 12 items from the short form of the Eysenck Personality Questionnaire (EPQ) (23) via self-report questionnaire.

#### Sample Selection

We have incorporated two novel strategies into our subject selection procedure, as described previously for this sample (24).

First, we have taken advantage of the extant literature that suggests shared genetic susceptibility among neuroticism, the anxiety disorders, and major depression (25–28). Starting with a total of 9270 twin subjects, we used multivariate structural equation modeling to estimate a latent genetic factor for neuroticism that is highly correlated with genetic susceptibility to major depression, generalized anxiety disorder, panic disorder, agoraphobia, and social phobia (see (28) for details). The factor derived from this analysis combines information across the correlated measures (phenotypes), like phenotypic factor analysis, but in this case uses shared genetic risk as the basis for this combination. Second, several authors have proposed using extreme phenotypic selection schemes to maximize the difference in information contained in a sample of subjects assessed on continuous measures, such as blood pressure or depression scores (29–31). However, unlike these selection schemes based only upon phenotypic extremes, the use of a genetically informative sample containing twins allows for the identification of subjects who are at the high and low extremes of genetic risk as well, as estimated by the twin pair’s score on the aforementioned genetic factor. Selecting subjects from the extremes of their underlying genetic risk factor should provide a powerful method for detecting genes of small effect expected to contribute to complex genetic phenotypes like psychiatric disorders. One member from each twin pair for whom DNA was available was selected as a case or control on the basis of scoring above the 80th or below the 20th percentile, respectively, of the genetic factor extracted from the aforementioned analysis. This produced a total sample size n = 1128 consisting of 589 cases (350 men, 239 women) and 539 control subjects (343 men, 196 women), of which 376 (196 men, 180 women) and 752 (497 men, 255 women) were used in stage 1 and stage 2, respectively. We note that this is more than just a selection on neuroticism, because, for example, some pairs score in the upper tail owing to high genetic loading on the clinical syndromes but might have relatively modest levels of neuroticism. Overall, the cases had a relatively modest level of neuroticism (mean raw neuroticism score of 6.3 (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 before the study, and informed consent was obtained from all subjects before data collection.

#### Table 1. Genetic Association Studies of COMT val158met Polymorphism (rs4680) with Anxiety Symptoms or Anxiety-Related Traits

<table>
<thead>
<tr>
<th>Study</th>
<th>Relevant Phenotype</th>
<th>Sample (n), Ethnicity</th>
<th>Associated Phenotype and Allele or Genotype (p)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoch et al. (67)</td>
<td>TPQ HA, EPQ N</td>
<td>149 Caucasian</td>
<td>HA: Met-Met (p &lt; 0.03)</td>
<td>(1) Associations limited to women</td>
</tr>
<tr>
<td>Olsson et al. (68)</td>
<td>CIS-R anxiety scales, NEO N</td>
<td>962 NA</td>
<td>“Episodic anxiety”: Met-Met (p = 0.02)</td>
<td>(1) Associations limited to women</td>
</tr>
<tr>
<td>Eley et al. (69)</td>
<td>NEO N</td>
<td>119 Caucasian</td>
<td>N: Met allele (p = 0.05)</td>
<td>(2) No association with N or “generalized anxiety”</td>
</tr>
<tr>
<td>Henderson et al. (62)</td>
<td>EPQ N/E/P, Bl, others</td>
<td>2327 Caucasian</td>
<td>No association with any measures</td>
<td>(1) Associations limited to women</td>
</tr>
<tr>
<td>McGrath et al. (4)</td>
<td>Phobic anxiety scale</td>
<td>1234 Caucasian</td>
<td>Phobia Scale: Val-Val (p = 0.01)</td>
<td>(1) COMT only tested in first stage sample (N = 848)</td>
</tr>
<tr>
<td>Stein et al. (7)</td>
<td>NEO N/E</td>
<td>497 Mixed</td>
<td>Low E: Met-Met (p = 0.02)</td>
<td>(2) No reported analyses by gender</td>
</tr>
<tr>
<td>Kim et al. (5)</td>
<td>TCI HA</td>
<td>286 Korean</td>
<td>HA: Val-Val (p = 0.003)</td>
<td>(1) Associations limited to women</td>
</tr>
<tr>
<td>Ishii et al. (70)</td>
<td>TCI HA</td>
<td>478 Japanese</td>
<td>HA: Met-Met (p = 0.059)</td>
<td>(1) Associations limited to women</td>
</tr>
<tr>
<td>Hashimoto et al. (71)</td>
<td>TCI HA</td>
<td>139 Japanese</td>
<td>HA: Met allele (p = 0.013)</td>
<td>(1) Gender-specific analyses were nonsignificant</td>
</tr>
</tbody>
</table>

TPQ, Tridimensional Personality Questionnaire; HA, harm avoidance; EPQ, Eysenck’s Personality Questionnaire; N, neuroticism; NA, Native American; CIS-R, Clinical Interview Schedule-Revised; NEO, Neuroticism Extraversion Openness personality inventory; E, extroversion; P, psychotism; Bl, behavioral inhibition; TCI, Temperament and Character Inventory.

Given the potential role of COMT in a broad range of internalizing phenotypes, in this study we sought to assess the potential association between COMT gene variants and shared genetic risk across a range of anxiety-related phenotypes in a large population-based sample. Specifically, we tested the val158met polymorphism as well as other markers that characterize the major allelic variation around the COMT locus, together with relevant haplotypes. Also, owing to the substantial proportion of reports that were specific to women, we also analyzed our results by gender.

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had a mean raw neuroticism score of 0.55 (Z score = -0.89). These statistics were similar across the two stages.

Genotyping

The DNA was extracted from buccal epithelial cells obtained via cytology brushes (32). The SNPs were genotyped by the 5′ nucleic acid cleavage assay (also called TaqMan method) (33). Reactions were performed in 96-well plates with 5 μL reaction volume containing 25 μL of 20X Assays-on-Demand SNP assay mix, 2.5 μL of Taqman universal polymerase chain reaction (PCR) master mix, and 5 ng of genomic DNA. Each 96-well plate contains samples for either cases or control subjects, and these are intercalated onto a single 384-well plate within a genotyping mix, 2.5 μL of Taqman universal polymerase chain reaction (PCR) master mix, and 5 ng of genomic DNA. Each 96-well plate contains samples for either cases or control subjects, 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 control subjects. The conditions for PCR were initial denaturing at 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. 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 Biosystems, Sunnyvale, California). Genotypes were scored by a Euclidian clustering algorithm developed in our laboratory 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.

COMT spans a 27-Kb interval on chromosome 22q11. The functional val158met polymorphism (hereafter referred to as rs4680), occurs in exon 4. Because several studies have questioned whether this polymorphism is the main susceptibility allele in the COMT region, we selected other SNP markers in an approximately 32-Kb interval around this gene with the aim to tag the major haplotypes observed in the Caucasian panel used by the HapMap project (34). We used the Tagger module of HAPLOVIEW 3.2 (35) with HapMap Phase II data, specifying aggressive tagging of 2- and 3-marker haplotypes and a threshold of $r^2 = .7$. This provided 8 tag SNPs (including rs4680) that captured the 16 alleles with minor allele frequency (MAF) > .05 in that interval with $r^2 > .73$. We added SNPs rs737865 and rs165599, given their potential relevance as indicated by prior studies of this gene (7,8), totaling 10 SNPs in and around the COMT locus for genotyping (see Results).

Statistical Analysis

We used a 2-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 with the LGA972 program (36) to achieve 80% power to detect markers that explained 1% of the variance of the liability distribution while controlling the false discovery rate at .1 (37). With a phenotypic extreme selection strategy with thresholds at 20% and 80%, respectively, LGA972 indicated that we needed approximately 350 subjects in the stage 1 and 1000 in the stage 2 sample. As described in the preceding text, we used somewhat smaller numbers than this in stage 2, but our selection was based on extremes of genetic, not phenotypic, factor scores, which should generally provide higher power to detect genetic effects, but this is not simple to estimate. If any of the markers genotyped in stage 1 met the screening $p$ value threshold of .1 or less, they were then also tested in the stage 2 sample.

We used Pearson’s $χ^2$ tests to test for allelic or genotypic differences by marker between cases and control subjects, separately by stage to check for consistency of results across the two stages. We used the program PEDSTATS (38) to test for Hardy-Weinberg equilibrium (HWE) violations for each marker. We used HAPLOVIEW 3.2 (35) to characterize linkage disequilibrium (LD) between the markers in our sample. Case-control haplotype association analyses were performed with the Coca-phase module of the UNPHASED program, version 2.4 (39). UNPHASED uses the expectation-maximization algorithm (40) to estimate the haplotypes and their frequencies. We used the program PHASE, version 2.1 (41,42), to reconstruct most likely multi-marker haplotypes for each subject for use in post hoc regression analyses when there were not one-to-one relationships between marker genotypes and haplotypes. We note that, despite the suggestive name of the software, we can only estimate haplotypes of unknown phase, given the case-control nature of our data. We performed each of these analyses for men and women together and separately.

For those markers or haplotypes that were consistently significant in each stage, we performed an overall analysis by combining data from both stages. A complication is that markers are selected for genotyping in stage 2 conditional upon their $p$ values in stage 1, so the test in stage 2 for any particular marker is not independent from that in stage 1. Assuming the conventional $χ^2$ distribution for the test statistic would result in a considerable increase in the number of false discoveries (43,44). To perform accurate tests on the combined data, we therefore used a different test statistic distribution that we derived previously (45,46).

The risk of false discoveries is considerable in candidate gene studies (47–49). To better assess this risk, we estimated the q-value for each marker genotyped in stage 2, which can be interpreted as the probability that a marker identified as significant is a false discovery (50–52). To estimate the q-values, one needs to know the prior probability that the marker has an effect as well as the effect size of the marker. Because the prior probability cannot be estimated reliably when only a relatively few markers are genotyped, we assumed a range of possible values for our calculations. Also, markers that, owing to sampling fluctuations, have a larger effect size are more likely to be selected as significant in stage 1. To estimate the effect size (odds ratio), we used data from stage 2 only, because the estimate tends to approach the true effect size in an independent sample (53).

Results

Association Testing

The genotype and allele frequencies and results of $χ^2$ association tests for the 10 COMT markers genotyped in stage 1 are listed in Table 2. The $p$ values are shown for the entire stage 1 sample and broken down by gender to indicate from which group significance might derive. To conserve space and simplify the table, the genotype and allele frequencies are only shown for the combination of men and women (the values broken down by gender are available upon request). All markers except three were in HWE; markers 5 and 10 showed modest deviations from HWE ($p = .002$ and $p = .025$, respectively) in cases only, whereas marker 7 showed more severe deviation ($p = .0006$) in both cases and control subjects. Figure 1 depicts the relative positions of these markers with respect to the intron-exon structure of the COMT gene.

Linkage disequilibrium information for these markers in our stage 1 sample is also provided in Figure 1 (with D′ and $r^2$). To better understand the LD...
Figure 1. Ten single nucleotide polymorphism markers genotyped across the COMT locus, with exons and untranslated regions as indicated. Linkage disequilibrium data (D’) and haplotype block pattern from HAPLOVIEW are displayed for these markers in the stage 1 sample.

Table 2. COMT Individual Marker Association Results for Stage 1

<table>
<thead>
<tr>
<th>Marker</th>
<th>Marker ID (dbSNP)</th>
<th>Alleles (major)</th>
<th>Group</th>
<th>Genotypes (%)</th>
<th>Genotypic p Value</th>
<th>Alleles (%)</th>
<th>Allelic p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A1/A1</td>
<td>A1/A2</td>
<td>A2/A2</td>
<td>All</td>
</tr>
<tr>
<td>1</td>
<td>rs2020917</td>
<td>T/C</td>
<td>Cases</td>
<td>52.4</td>
<td>40.1</td>
<td>7.5</td>
<td>.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>56.4</td>
<td>37.2</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>rs737865</td>
<td>A/G</td>
<td>Cases</td>
<td>52.7</td>
<td>40.9</td>
<td>6.4</td>
<td>.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>58.1</td>
<td>37.0</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>rs740603</td>
<td>A/G</td>
<td>Cases</td>
<td>28.6</td>
<td>48.7</td>
<td>22.7</td>
<td>.015*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>17.8</td>
<td>62.7</td>
<td>19.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>rs4680</td>
<td>A/G</td>
<td>Cases</td>
<td>25.5</td>
<td>33.2</td>
<td>41.3</td>
<td>.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>34.1</td>
<td>50.5</td>
<td>15.4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>rs464316</td>
<td>T/C</td>
<td>Cases</td>
<td>59.6</td>
<td>29.8</td>
<td>10.6</td>
<td>.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>62.8</td>
<td>30.8</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>rs165774</td>
<td>A/G</td>
<td>Cases</td>
<td>45.4</td>
<td>45.3</td>
<td>9.3</td>
<td>.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>46.9</td>
<td>44.1</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>rs174696</td>
<td>T/C</td>
<td>Cases</td>
<td>66.1</td>
<td>25.3</td>
<td>8.6</td>
<td>.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>55.7</td>
<td>34.6</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>rs174699</td>
<td>T/C</td>
<td>Cases</td>
<td>88.8</td>
<td>11.2</td>
<td>.0</td>
<td>.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>89.2</td>
<td>10.8</td>
<td>.0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>rs9322377</td>
<td>T/C</td>
<td>Cases</td>
<td>68.6</td>
<td>28.7</td>
<td>2.7</td>
<td>.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>74.5</td>
<td>24.5</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>rs165599</td>
<td>A/G</td>
<td>Cases</td>
<td>48.1</td>
<td>46.5</td>
<td>5.4</td>
<td>.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Controls</td>
<td>46.8</td>
<td>46.3</td>
<td>6.9</td>
<td></td>
</tr>
</tbody>
</table>

Cases (n = 188), control subjects (n = 188), men (M) (n = 196), women (F) (n = 180), and together (All).

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

structure, we constructed haplotype blocks with the default Confidence Interval procedure (54) in HAPLOVIEW 3.2. As indicated in Figure 1, pairs of markers [1, 2, 5, 6], and [9, 10] are in high LD, with the latter two pairs being part of haplotype blocks. This is roughly consistent with data from HapMap. We note that LD is quite modest across the COMT gene overall. This would generally disfavor performing haplotype association tests for widely spaced markers across the gene. However, prior analyses in schizophrenia (8) and personality (7) (among other phenotypes) have produced significant findings with combinations of rs4680 with markers 2 and 10 in this locus. On this basis, we created haplotypes from relevant marker combinations. Markers 4, 7, and 9 met threshold criteria of allelic p value < .1 in our stage 1 sample; we genotyped these plus markers 2 and 10 in stage 2 (Table 3). We chose these latter two markers to analyze relevant haplotypes in both stages implicated in prior studies (note: whereas marker 3 displayed significant association in the genotypic analysis in stage 1, particularly for women, this differed from the results of the allelic analysis; this, together with the results of exploratory analyses that suggested that this marker does not contribute to haplotype association, led us to decide not to genotype it in stage 2). There are, comparing Tables 2 and 3, no clearly consistent single marker associations across stages. Although marker 7 showed marginal association in stage 2, it continued to suffer from severe deviations from HWE in the total sample (p = 1.3e-5); the other four markers did not show HWE deviations when the entire sample was considered. Therefore, we excluded this marker from further analyses.

In Table 4, we present the results, by stage, of haplotype association tests for several relevant marker combinations as calculated with the Cocaphase module of UNPHASED. The haplotypes constructed from markers (2-4-10) correspond to those analyzed by Shifman et al. (8) for schizophrenia and Stein et al. for personality (7); it did not produce a pattern of association that was replicated across stages. However, closer inspection of these results suggested that the G-A haplotype of the simpler combination of markers 4 and 10 within that three-marker combination was driving associations that were seen, particularly in women. Marker 2, further away from and in low LD with the other two markers, seemed to be diluting the signal. This was supported by the stronger, more consistent association seen with the G-A haplotype from markers 4 and 10, which is also more common than the three-marker haplotypes examined. This association is confined to the women.

On the basis of these findings, we sought to analyze the effects of our best candidate markers and haplotypes in the combined stage 1 + stage 2 female sample. For this purpose, we chose marker 4 (rs4680), owing to its relevance from prior studies, and our G-A haplotype from markers 4 and 10. We used the program PHASE, version 2.1 (41,42), to reconstruct these
two-marker haplotypes for all individuals in our sample. To maximize confidence in the estimated haplotypes, we used the most likely pairs of haplotypes for each subject and discarded data from subjects if haplotype probabilities did not exceed .8.

Table 5 displays the p values for female subjects pooled across both stages and the stage 2 case-control odds ratios for marker 4 (rs4680) and the G-A haplotype. We estimated a roughly 24% increase in risk associated with the G (val) allele of marker 4 and almost double the risk associated with the G-A haplotype from markers 4 and 10. Table 5 also displays the estimated false-discovery rate q, that is, the global probability that the combined results for each particular marker or haplotype occurred purely by chance, as a function of the assumed prior probability of true discovery, p0. Whereas q-values are above 50% for marker 4, those for the G-A haplotype are all below 6%. This suggests that, when the corresponding p value (1.97e-5) is used to declare significance, the expected proportion of false discoveries among significant tests would be 6% or less.

In post hoc analyses we explored whether these findings for COMT, on the basis of individual differences in genetic factor
scores, were a result of associations with specific phenotypes within our total sample. With Cochran-Mantel-Haenszel tests in the FREQ procedure of SAS (55), we detected significant associations (non-zero correlation) between marker 4 and the G-A haplotype and most of our measured phenotypes, as shown in Table 6. For simplicity, we only display the results for women, because no associations were observed for men. We note that these analyses extend beyond our original hypotheses and do not control for such factors as multiple testing or correlated phenotypes. The relationship between mean N score and number of copies of the G-A haplotype in women is graphed in Figure 2.

Population Stratification
We explored, as a potential concern for any case-control association study, the possibility that these results were obtained spuriously, owing to population stratification with three methods. First, with self-reported ancestry data from this entirely Caucasian sample, we did not detect any evidence of ethnic background differences between cases and control subjects. Second, with a set of 24 unlinked markers chosen for convenience from experiments on other candidate loci, the software STRUCTURE (56) found no significant genetic subpopulations. Finally, with the method of Genomic Control (57) on the same 24 unlinked markers, we found no evidence of variance inflation that could be attributed to stratification. In addition to these investigations in the current sample, Sullivan et al. (58) found no evidence for stratification with 16 unlinked microsatellite markers in a case-control study of nicotine dependence in a different subset of our twin sample (n = 900).

Discussion
In this study, we sought to test whether the COMT gene is associated with susceptibility to human anxiety spectrum phenotypes, including neuroticism, a range of anxiety disorders, and major depression. This susceptibility was indexed by a latent genetic factor common to these phenotypes, the score of which we derived from multivariate twin modeling and subsequently used to select subjects at the extremes of genetic risk. We entered the resulting sample of 589 cases and 539 control subjects into a two-stage association study in which markers from the candidate locus were screened in stage 1, the positive results of which were tested for replication in stage 2. Because prior studies highlight the potential importance of analyzing haplotypes across this gene, we tested several markers in addition to the functional val158met polymorphism (rs4680).

Out of a total of ten markers tested in the COMT gene, three met the threshold criterion in stage 1 of p < .1 for genotyping in stage 2. These three, plus two others selected on the basis of haplotype analyses from other studies, were then genotyped in the stage 2 sample. Although there were no consistent associations seen across the two stages for individual markers, rs4680 showed marginally significant association in women when data from both stages were combined. The G-A haplotype formed from the G (val) allele of marker rs4680 together with the A allele of rs165599 showed significant association in each stage and in the entire female sample (p = 1.97e-5). Furthermore, this haplotype more significantly predicted than rs4680 alone each of the specific psychiatric phenotypes relevant for our study (again, in women only), including neuroticism (p = 1e-5), major depression (p = 2e-4), and several anxiety disorders. We tested for and could not detect any evidence that these were spurious association signals due to population stratification. However, this latter analysis was limited to only 24 markers, which might be insufficient to detect modest levels of stratification (59).

Declaring a positive association in a candidate gene study is not without controversy (60). Although no individual marker, including rs4680, displayed consistent association across both stages, several haplotypes did, specifically in women. Although this is not a formal replication by recent standards (60), because both stages derive from the same overall sample of twins, it nonetheless provided justification for jointly analyzing data across the two stages to maximize power (61). A naïve application of Bonferroni correction (i.e., dividing a significance threshold of p = .05 by the total number of tests performed) might not be appropriate here, given the non-independence between tests.

<table>
<thead>
<tr>
<th>Marker or Haplotype</th>
<th>q-Values(^a)</th>
<th>Allelic Odds Ratio(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4680 (G allele)</td>
<td>(p_0 = .95)</td>
<td>(p_0 = .99)</td>
</tr>
<tr>
<td>G-A Haplotype</td>
<td>(p_0 = .999)</td>
<td>(p = 1.97e-5)</td>
</tr>
</tbody>
</table>

\(^a\)False discovery rate for three values of prior probability of true effect \(p_0\).
\(^b\)Derived from stage 2 data only.

Table 5. Allele-Based Test Statistics for COMT Marker rs4680 or Two-Marker (rs4680, rs165599) G-A Risk Haplotype for Entire Female Sample Pooled Across Both Stages (n = 435)

Figure 2. Graph of the relationship between mean neuroticism (N) score and number of copies of two-marker (rs4680, rs165599) G-A risk haplotype identified in COMT gene.
of markers in LD with each other and overlapping haplotypes constructed from them. Also, in general there are practical and theoretical limitations to the Bonferroni correction (44). Therefore, we estimated the probability that our a priori candidate polymorphism (val158met) and our best haplotype were false discoveries (q-values). Examining Table 5, we see that, even though rs4680 showed association at p = .0094 level of significance, this also has high probability of being a false discovery. However, the G-A haplotype formed from markers rs4680 and rs165599 not only showed highly significant association in women but our q-value estimates suggest that this is unlikely a false discovery.

The fact that our identified high-risk haplotype had significant allelic differences in our original sample selected for shared genetic risk for internalizing phenotypes and significantly predicted each of the individual phenotypes themselves suggests that COMT or another locus in LD with this haplotype acts as a broad psychiatric risk factor. Consistent with studies in other phenotypes, COMT haplotypes containing marker rs4680 confer a greater effect on risk than the functional val158met polymorphism alone, although exactly which allele corresponds to increased risk seems to depend upon phenotype studied and ethnic makeup of the sample, as reviewed in the first part of this report. Our high-risk haplotype contains the G (val) allele of rs4680. This is consistent with prior studies of panic disorder in Caucasian samples (10) as well as a large European study of early-onset major depressive disorder (13). However, this seems to be somewhat at odds with a number of prior studies of neuroticism and other anxiety-related traits and symptoms (see Table 1). Although several of the smaller studies reported marginally significant associations with the met allele or met-met genotype, the two largest studies either found no association with this locus (62) or association with the opposite (val) allele (4). Among those studies, only Stein et al. (7) examined the effects of markers other than rs4680. In a mixed ethnic sample of 497 college students, they tested the three markers (rs737865, rs4680, and rs165599—corresponding to our markers 2, 4, and 10) and related haplotypes previously found to be highly associated with schizophrenia in a sample of Ashkenazi Jews (8). They found marginally significant associations of the met-met genotype with low extroversion (a trait related to social phobia) and high neuroticism in their female subjects and variously associated three-marker haplotypes depending upon whether extroversion and neuroticism were analyzed via median split of their sample or as continuous traits. Specifically, whereas their strongest reported association was between the G-A-A haplotype and dichotomous neuroticism in women (p = .005), associations were also seen in their results tables for haplotypes containing the G-A alleles of markers 4 and 10 and both phenotypes. For comparison, we note that Shifman et al. (8) reported that the G (val) allele was modestly associated with schizophrenia in men, with the strongest associations from the three-marker haplotype G-G-G.

Given the approximately twofold increased risk for anxiety and depressive disorders in women compared with men, our identification of a haplotype that broadly increases risk for internalizing disorders in women only is intriguing. However, the mechanism by which this haplotype increases risk in women but not men is yet to be discerned. In our sample, men and women did not, on average, significantly differ in the frequencies of this high-risk haplotype. One hypothesis is that differing levels of estrogenic hormones between women and men interact with this haplotype to differentially affect risk. Multi-marker COMT haplotypes have been shown to modulate expression of this gene in the brain (6), possibly in a gender-specific fashion (63). Furthermore, the COMT gene has an estrogenic response element in its promoter region, so certain haplotypes containing this could produce differential expression of COMT depending upon estrogen levels (64). As a key enzyme in the conjugation of catecholestrogens (65), COMT genotype also affects circulating levels of estrogen (66), suggesting a complex bidirectional interaction between estrogenic hormones and COMT haplotypes that could, presumably, impact psychiatric risk in a gender-specific manner.

In conclusion, we have identified genetic variations in the COMT gene that significantly associate with a range of internalizing psychiatric phenotypes. In particular, in agreement with prior studies that have examined multiple markers across this locus, the val158met polymorphism alone is unable to fully explain this association. Rather, haplotype analyses suggest that variations in or around COMT in LD with this polymorphism increase susceptibility for women, but exactly where or on what level these variations contribute to this risk remains unclear. Future studies of COMT would thus benefit from the inclusion of other markers in addition to the val158met polymorphism and the performance of gender-specific analyses.

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