

Direct, indirect and pleiotropic effects of candidate genes on internalizing disorder psychopathology

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Background. Twin studies of internalizing disorders suggest that their high co-morbidity is partially explained by shared genetic risk. Few studies have investigated pleiotropic effects of well-validated candidate genes across phenotypes.

Method. Subjects were 928 Caucasian patients who presented to an out-patient clinic specializing in the assessment and treatment of anxiety and mood disorders. We constructed latent dimensional phenotypes across the internalizing spectrum (neuroticism, extraversion, depression, generalized anxiety, panic/agoraphobia, social phobia, post-traumatic stress, and obsessions–compulsions) by combining diagnostic criteria with other clinical indicators. We selected multiple variants in four evidence-based candidate genes (*SLC6A4*, *COMT*, *GAD1*, *RGS2*) with previously reported effects on several of these phenotypes. We conducted genetic association testing of their direct and indirect effects as well as gene × stress interactions (G × E).

Results. We detected 19 nominally significant main effect associations for the 10 polymorphisms tested among the eight phenotypes (24%). These were generally phenotype non-specific, showing pleiotropic effects across multiple domains. The majority of observed sharing was between depression, panic disorder, and post-traumatic stress disorder. Some of these were best explained by mediational models in which genes increase liability for disorders indirectly via their effects on temperament. Limited G × E effects were detected between variants in *SLC6A4* and both panic/agoraphobia and post-traumatic stress.

Conclusions. Examining just a few candidate genes for their potential roles in internalizing phenotypes, we found moderate support for the shared effects of several polymorphisms. These findings highlight the richness and complexity by which genes potentially contribute to psychopathology via pleiotropy, moderation by stress, and mediation by temperament.

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Introduction

Anxiety disorders, like generalized anxiety disorder (GAD), panic disorder (PD) and phobias, are common, disabling conditions with substantial lifetime prevalence (Kessler *et al.* 2005). They tend to persist throughout the life course and exhibit strong co-morbidity with each other and with other internalizing disorders like major depressive disorder. Twin and family studies implicate genetic factors in their etiology, with moderate levels of familial aggregation and heritability (Hettema *et al.* 2001). Dimensions of temperament such as neuroticism and extraversion are related to internalizing liability

and co-morbidity (Andrews *et al.* 1990; Bienvenu *et al.* 2001; Khan *et al.* 2005). Twin studies suggest that some genetic factors non-specifically increase risk across these related phenotypes (genetic pleiotropy) (Jardine *et al.* 1984; Hettema *et al.* 2006b; Kendler *et al.* 2007). Cross-disorder effects of novel candidate genes have been reported in recent psychiatric genome-wide association studies (GWAS) (Cross-Disorder Group of the Psychiatric GWAS Consortium, 2013). Thus, studying these phenotypes in a coordinated manner is important for elucidating the effects of specific susceptibility genes.

The twin studies cited above support shared (specifically, correlated) genetic factors between various internalizing phenotypes. One possible mechanism for this, at least regarding the relationships between temperament and internalizing disorders, is mediation. That is, genes might indirectly increase liability for anxiety and depressive disorders via genetic variation

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of underlying traits of temperament such as neuroticism. In a longitudinal twin study of neuroticism and major depression, Kendler *et al.* (1993) found that a latent genetic factor underlying neuroticism was the largest contributor to later risk of major depression. To our knowledge, only one prior study investigated this hypothesis for a specific gene. Munafò *et al.* (2006) reported that observed associations between genotype at the serotonin transporter promoter length polymorphism (5-HTTLPR), neuroticism and lifetime major depression could be partially explained by a mediational model, in which neuroticism accounted for 42.3% of the effect of 5-HTTLPR genotype on lifetime major depression.

With heritability around 50%, environmental factors like stressful life events are as important as genetic factors in the etiology of internalizing disorders. Besides their main effects, genes and environment probably interact ($G \times E$) such that some genes make an individual more or less sensitive to the pathogenic effects of stress. Prior studies of specific polymorphisms in a few candidate genes have examined $G \times E$ effects for single phenotypes [primarily depression and post-traumatic stress disorder (PTSD); for recent reviews, see Mandelli & Serretti (2013) and Digangi *et al.* (2013), respectively]. To our knowledge, no studies have tested for these effects across multiple internalizing disorder outcomes.

The aims of this study are to: (1) identify the differential and pleiotropic effects of several validated candidate genes across a wide range of internalizing disorder phenotypes; (2) replicate prior associations of specific variants with particular disorders; (3) test for mediation of the effects of genes on disorders through temperament; and (4) investigate how stress moderates the risk patterns associated with these genes. A particularly novel aspect of this inquiry is that candidate genes are included in latent variable measurement models as predictors of internalizing disorder and temperament constructs. In such models, statistical power can be fostered considerably by using dimensional outcomes and multiple indicators of each variable.

Method

Participants

The sample consisted of 928 Caucasian/non-Latino patients who presented to the Center for Anxiety and Related Disorders at Boston University, an out-patient clinic specializing in the assessment and treatment of anxiety and mood disorders. Women constituted the larger proportion of the sample (59.9%); average age was 32.05 years (s.d. = 11.85, range = 18–69 years). Diagnoses were established with the Anxiety

Disorders Interview Schedule for DSM-IV: lifetime version (ADIS-IV-L; Di Nardo *et al.* 1994), a semi-structured interview designed to ascertain reliable diagnosis of the DSM-IV anxiety, mood, somatoform and substance use disorders and to screen for the presence of other conditions (e.g. psychotic disorders). The ADIS-IV-L provides dimensional assessment of the key and associated features of disorders (0–8 ratings); these features are dimensionally rated regardless of whether a formal DSM-IV diagnosis is under consideration. A previous reliability study of the ADIS-IV-L indicated good-to-excellent interrater agreement for current disorders (range of κ 's = 0.67–0.86) except dysthymia ($\kappa = 0.31$) (Brown *et al.* 2001). The rates of current clinical disorders occurring frequently in the sample were: social phobia (SOC) (47.6%); mood disorders (i.e. major depression, dysthymic disorder, depressive disorder not otherwise specified; 31.7%); GAD (33.1%); PD with (PD/A) or without agoraphobia (27.6%); obsessive-compulsive disorder (OCD) (13.1%); and specific phobia (13.8%). The study protocol (interviews, questionnaires, DNA collection) was approved by the Institutional Review Board of Boston University, Charles River campus, and all study procedures (e.g. informed consent) were performed in accordance with approved guidelines and regulations.

Latent phenotypes and indicators in the genetic models

As in previous studies (e.g. Brown, 2007; Brown & Naragon-Gainey, 2013), each of our internalizing outcomes was represented by a latent construct with multiple measured indicators, producing error-free dimensional phenotypes incorporating greater information content than provided by diagnosis alone (e.g. individual differences in severity). In addition to two temperaments (neuroticism, extraversion), six DSM-IV disorder latent constructs were examined in the genetic models: depression, GAD, OCD, SOC, PD/A and PTSD.

Temperament

Latent variables corresponding to the temperaments of neuroticism and extraversion were defined by subscales from the NEO-Five Factor Inventory (Costa & McCrae, 1992), the short form version of the Eysenck Personality Questionnaire (Eysenck & Eysenck, 1975) and the Behavioral Inhibition/Behavioral Activation Scales (Carver & White, 1994).

Depression

A latent variable of unipolar depression was formed using two questionnaire indicators and an ADIS-IV-L

clinical rating composite: (a) Beck Depression Inventory-II (BDI-II; Beck *et al.* 1996); (b) depression scale of the 21-item version of the Depression Anxiety Stress Scales (Lovibond & Lovibond, 1995; see also Antony *et al.* 1998); and (c) the ADIS-IV-L dimensional ratings of the nine symptom criteria of DSM-IV major depression (0 = 'none' to 8 = 'very severe'). In accord with procedures from previous studies (e.g. Brown, 2007; Brown & Rosellini, 2011), the BDI-II was scored using the 10 items that load on a cognitive/affective factor (items 1–9, 13) that are more specific to the unipolar mood disorders.

GAD

A factor of DSM-IV GAD was created in part by using three ADIS-IV-L dimensional rating measures (all 0–8 scales): excessiveness of worry in eight areas (e.g. finances, minor matters); a composite score of the six symptoms comprising the GAD-associated symptom criterion, and a single clinical rating of patients' difficulty controlling worry. An additional indicator of the GAD factor was the Penn State Worry Questionnaire (Meyer *et al.* 1990), a 16-item self-report measure of chronic worry.

SOC

Two questionnaires and one ADIS-IV-L clinical rating measure were used as indicators of the SOC latent variable: (a) social phobia scale of the Albany Panic and Phobia Questionnaire (APPQ-S; Rapee *et al.* 1994–1995; Brown *et al.* 2005); (b) the Social Interaction Anxiety Scale (Mattick & Clarke, 1998); and (c) a sum composite of patients' fear of 13 social situations (e.g. initiating a conversation, participating at meetings/classes) rated by the clinician during the ADIS-IV-L (0 = 'no fear' to 8 = 'very severe fear').

OCD

The OCD latent variable was represented by two clinical ratings and one questionnaire. The clinical rating indicators were: (a) a sum composite of ADIS-IV-L dimensional ratings of persistence/distress associated with nine common obsessions (e.g. doubt, contamination); and (b) a sum composite of ADIS-IV-L dimensional ratings of the frequency of six compulsions (e.g. washing, checking). The self-report indicator was the total score of the Obsessive–Compulsive Inventory–Revised (Foa *et al.* 2002).

PD/A

Two questionnaires and two ADIS-IV-L clinical rating measures were used to form a latent variable representing DSM-IV PD/A: (a) the interoceptive fear and

agoraphobia scales of the APPQ (APPQ-I and APPQ-A; Rapee *et al.* 1994–1995); (b) a sum composite of dimensional ratings of the three symptoms comprising DSM-IV criterion A2 (worry/change in behavior); and (c) a sum composite of the ADIS-IV-L dimensional ratings of situational avoidance of or escape from 22 agoraphobic situations (agoraphobia).

PTSD

The latent variable of DSM-IV PTSD was formed using ADIS-IV-L dimensional ratings of the 17 symptoms comprising DSM-IV criteria B, C and D. The indicators were symptom composites created on the basis of the four-factor model presented in Simms *et al.* (2002): (a) intrusions – criteria B1 through B5; (b) avoidance – criteria C1 and C2; (c) dysphoria – criteria C3 through C7, and criteria D1 through D3; and (d) hyperarousal – criteria D4 and D5.

Measure of stress

Chronic life stress was assessed at intake using the UCLA Life Stress Interview (UCLA-LSI; Hammen *et al.* 1987). The UCLA-LSI is a semi-structured interview that assesses stress occurring over the prior 6 months in eight domains: social life, romantic relationships, family, work, school, finances, health of self, and health of others. The UCLA-LSI defines chronic stress as a strain lasting at least 6 months. Interviewers made a chronic stress rating for each domain on a five-point scale (1 = exceptionally positive circumstances to 5 = extremely adverse circumstances) using descriptive behavioral anchors. The chronic stress variable used in the analyses was the sum composite of the eight stress domain ratings (see Brown & Rosellini, 2011).

Candidate gene selection

We selected a small number of candidate genes based on evidence from multiple extant studies showing significant main effects of the gene on one or more of the relevant internalizing phenotypes studied. For some genes, associations with several variants have been previously reported for the same or related phenotypes. We chose single nucleotide polymorphisms (SNPs) and, where applicable, multimarker haplotypes, in the following four genes that have demonstrated specific prior associations: (a) the catechol O-methyl-transferase gene (*COMT*); (b) the glutamic acid decarboxylase 1 gene (*GAD1*); (c) the gene coding for regulator of G-protein signaling 2 (*RGS2*); and (d) the serotonin transporter gene (*SLC6A4*). For *COMT*, we selected two SNPs: rs4680, coding the well-known val158met functional polymorphism implicated in various psychiatric phenotypes (Craddock *et al.* 2006); and

rs165599, which together with rs4680, forms a 2-marker haplotype which we previously reported as strongly associated with female-specific internalizing genetic risk in another sample (Hettema et al. 2008), suggesting a female-specific subanalysis for *COMT*. For *GAD1*, we selected four SNPs that form a haplotype reportedly associated with shared genetic liability across several anxiety disorders in two studies (Hettema et al. 2006a; Donner et al. 2012). For *RGS2*, we selected two representative SNPs from several that had previously shown associations with multiple human anxiety phenotypes, including PD (Leygraf et al. 2006), GAD (Koenen et al. 2009) and extraversion (Smoller et al. 2008).

For *SLC6A4*, we selected several distinct sets of polymorphisms across the gene based upon prior reports: two SNPs previously associated with PD, rs3813034 (Gyawali et al. 2010) and rs140701 (Strug et al. 2010); two SNPs in high linkage disequilibrium (LD) with each other, rs6354 and rs2020936, associated with multiple anxiety and depression phenotypes (Wray et al. 2009); and two SNPs, rs4251417 and rs2020934, the C-A haplotype of which reportedly tags ($r^2 = 0.72$) the short allele of the serotonin transporter promoter length polymorphism 5-HTTLPR (Wray et al. 2009), for which many, sometimes inconsistent, reports of association with internalizing phenotypes exist.

Genotyping

DNA was extracted from either blood or saliva samples using standard procedures. SNPs were genotyped by the TaqMan method (Livak, 1999): polymerase chain reaction (PCR) was performed in 96-well plates with 5 μ l reaction volume containing 0.25 μ l of 20X Assays-on-Demand™ SNP assay mix, 2.5 μ l of TaqMan universal PCR master mix, and 5 ng of genomic DNA. The conditions for PCR were initial denaturizing at 95°C for 10 min, followed by 40 cycles of 92°C for 15 s 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). Genotypes were scored by a Euclidian clustering algorithm developed in our laboratory and checked for deviations from Hardy–Weinberg equilibrium. To ensure the quality of genotyping, negative control samples were included in each plate, and we performed duplicate genotyping on a subset of samples.

Statistical analysis

The raw phenotypic data were analysed using a latent variable software program and maximum likelihood (ML) minimization functions (Mplus 7.11) (Muthén &

Muthén, 2010). Although negligible, missing data on phenotypic measures were accommodated by ML (see Allison, 2003). Goodness of fit of the models was evaluated using the root mean square error of approximation (RMSEA) and its 90% confidence interval (CI), the Tucker–Lewis index (TLI), the comparative fit index (CFI) and the standardized root mean square residual (SRMR). Acceptable model fit was defined in part by the criteria forwarded by Hu & Bentler (1999): RMSEA values close to 0.06 or below (90% CI upper limit close to ≤ 0.06 , non-significant), CFI and TLI values close to 0.95 or above, and SRMR values close to 0.08 or below. The acceptability of the models was further evaluated by the presence/absence of salient localized areas of strains in the solutions (e.g. modification indices), and the strength and interpretability of the parameter estimates. All measurement models fit the data well by these guidelines and the factor loadings for the indicators of each phenotype construct were salient in magnitude (range of standardized loadings = 0.51–0.92) and statistically significant (all p 's < 0.001). The disorder constructs were weakly to moderately intercorrelated (smallest $r = 0.06$ between OCD and PTSD, largest $r = 0.51$ between GAD and depression). All disorder constructs were significantly related (p 's < 0.001) to neuroticism (range of r 's = 0.14–0.75); consistent with previous research (e.g. Naragon-Gainey et al. 2013), only SOC and depression had salient relationships with extraversion (r 's = -0.78 and -0.39 , respectively).

After good-fitting latent variable measurement models were established, the direct effects of genotypes on phenotypes were tested by regressing the latent variable phenotypes onto the genes. For mediational models, the size and significance of gene \rightarrow temperament \rightarrow disorder effects were estimated using the MODEL INDIRECT feature of Mplus. In addition to the indirect effect, the direct effect of the gene on the phenotype was freely estimated in these models (e.g. to evaluate full versus partial mediation). Gene–stress interaction effects were examined in hierarchical structural regression models where the latent variable phenotype was regressed onto the gene and chronic stress variable in the first analysis (main effects model), and the gene, chronic stress, and gene \times chronic stress product term were specified as predictors of the latent variable phenotype in the second analysis (moderation model).

Ethical standards

All procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Table 1. Tests of association between internalizing phenotypes and candidate gene polymorphisms – main and interaction effects with stress ($G \times E$)

Gene	Variant	Effect	Phenotype							
			N	E	GAD	OCD	DEP	SOC	PD/A	PTSD
<i>SLC6A4</i>	rs3813034	Main	0.476	0.939	0.055*	0.933	<0.001*	0.170	0.093*	0.005*
		$G \times E$	0.386	0.142	0.099*	0.274	0.622	0.174	0.001*	0.002*
	rs140701	Main	0.455	0.418	0.041*	0.551	0.001*	0.347	0.148	0.009*
		$G \times E$	0.456	0.474	0.326	0.173	0.908	0.537	0.015*	0.005*
	rs6354	Main	0.636	0.626	0.266	0.786	0.026*	0.663	0.087*	0.962
		$G \times E$	0.613	0.384	0.905	0.929	0.418	0.332	0.142	0.007*
	rs2020936	Main	0.209	0.562	0.017*	0.572	0.002*	0.668	0.017*	0.413
		$G \times E$	0.511	0.452	0.897	0.746	0.445	0.347	0.070*	0.001*
	5-HTTLPR(s) ^a	Main	0.078*	0.506	0.241	0.462	0.008*	0.117	0.209	0.048*
		$G \times E$	0.839	0.053*	0.528	0.779	0.905	0.479	0.828	0.188
<i>COMT</i> ^b	rs4680	Main	0.021*	0.016*	0.378	0.152	0.043*	0.070*	0.462	0.934
		$G \times E$	0.063*	0.175	0.813	0.686	0.249	0.454	0.289	0.160
	2-marker haplotype ^c	Main	0.013*	0.016*	0.228	0.418	0.033*	0.010*	0.394	0.823
		$G \times E$	0.024*	0.299	0.709	0.672	0.209	0.616	0.710	0.093*
<i>GAD1</i>	4-marker haplotype ^d	Main	0.004*	0.105	0.104	0.605	0.128	0.108	0.043*	0.004*
		$G \times E$	0.148	0.081*	0.464	0.402	0.055*	0.551	0.138	0.357
<i>RGS2</i>	rs4606	Main	0.564	0.958	0.505	0.432	0.386	0.247	0.554	0.175
		$G \times E$	0.689	0.292	0.881	0.578	0.251	0.635	0.180	0.382
	rs6428136	Main	0.610	0.875	0.426	0.576	0.345	0.367	0.586	0.266
		$G \times E$	0.928	0.239	0.962	0.676	0.272	0.452	0.291	0.205

$G \times E$, Genes \times environment interaction; N, neuroticism; E, extraversion; GAD, generalized anxiety disorder; OCD, obsessive-compulsive disorder; DEP, depression; SOC, social phobia; PD/A, panic disorder/agoraphobia; PTSD, post-traumatic stress disorder; *SLC6A4*, serotonin transporter; 5-HTTLPR(s), short variant of serotonin transporter promoter length polymorphism; *COMT*, catechol *O*-methyl-transferase; *GAD1*, glutamic acid decarboxylase 1; *RGS2*, regulator of G-protein signaling 2.

^a 5-HTTLPR(s) here is represented by the C-A haplotype of single nucleotide polymorphisms rs4251417 and rs2020934.

^b Results restricted to females only.

^c *COMT* 2-marker haplotype indicates the rs4680-rs165599 haplotype A-A.

^d *GAD1* 4-marker haplotype indicates the rs2241165-rs769407-rs3791851-rs3791850 haplotype A-C-G-C.

* p Values statistically significant or approaching significance ($p \leq 0.10$).

Results

Phenotype–genotype associations

DNA was of sufficient quality for genotyping of 928 subjects. All SNPs were successfully genotyped and passed quality-control metrics. Online Supplementary Fig. S1 depicts the LD pattern (r^2) obtained using Haploview (Barrett *et al.* 2005) for genotyped SNPs across the *SLC6A4* locus in our sample. There is high correlation between rs6354 and rs2020936 and modest correlation between rs3813034 and rs140701, suggesting potential overlap in any results for these pairs. Haplotype patterns for *COMT* and *GAD1* SNPs were consistent with those reported in our prior studies.

The results of the association analyses are presented in Table 1. The most significant ($p < 0.01$) main-effect associations were, by gene: (a) *SLC6A4* rs3813034 and rs140701 with depression and PTSD, rs2020936 and the short variant of 5-HTTLPR [5-HTTLPR(s)] with

depression; (b) *GAD1* 4-marker haplotype with neuroticism and PTSD. Of note, *RGS2* was not associated with any phenotype. Of the 80 variant–phenotype main effects tested, 19 (24%) had $p \leq 0.05$, suggesting that at least some of these associations are probably valid. (We note that, due to correlations between phenotypes and between markers in a gene, a Bonferroni-corrected p value would be overly conservative.) Remarkably, no significant associations were found between OCD and any of the candidate genes. Many, at least marginal, associations ($p \leq 0.1$) were observed: (a) all of the tested *SLC6A4* SNPs with depression and several with GAD, PD/A or PTSD; (b) 5-HTTLPR(s) with neuroticism, depression and PTSD; (c) the *COMT* haplotype with neuroticism, extraversion, depression and SOC; (d) the *GAD1* haplotype with neuroticism, GAD, PD/A and PTSD. Thus, while many possible associations were not detected, there is an overall pattern of broad sharing of genetic effects across phenotypes.

Table 2. Results of mediational modeling: gene → temperament → disorder

Gene	Variant	Temperament	Disorder	<i>p</i>
COMT ^a	rs4680	Neuroticism	DEP	0.008
	rs4680	Extraversion	DEP	0.008
	2-marker haplotype	Neuroticism	DEP	0.007
	2-marker haplotype	Extraversion	DEP	0.007
	2-marker haplotype	Neuroticism	SOC	0.016
	2-marker haplotype	Extraversion	SOC	0.005
GAD1	4-marker haplotype	Neuroticism	PD/A	0.015
	4-marker haplotype	Neuroticism	PTSD	0.032

COMT, Catechol *O*-methyl-transferase; DEP, unipolar depression; SOC, social phobia; GAD1, glutamic acid decarboxylase 1; PD/A, panic disorder/agoraphobia; PTSD, post-traumatic stress disorder.

^a Results restricted to females only.

Mediational models

We tested mediational models (gene → temperament → disorder) for the eight temperament–disorder pairs for which the same genetic variants showed zero-order association at $p \leq 0.05$. The results are displayed in Table 2. All analyses indicated significant indirect effects, and all but one suggested full mediation (the exception being GAD1 → neuroticism → PTSD, where both the direct and indirect effects of the gene on PTSD were significant). This supports the hypothesis that at least part of the mechanism by which these genes increase liability for disorders is indirectly via their effects on temperament.

Gene × stress interactions

We tested for the presence of gene–environment correlations before examining G × E interaction, as these can be confounded. All were small ($r < 0.1$) and for only one polymorphism [HTTLPR(s)] was it significantly different from zero ($r = -0.08$, $p = 0.02$). As indicated in Table 1, each of the SLC6A4 variants except for 5-HTTLPR(s) showed significant interactive effects with stress (G × E) for either PD/A or PTSD. The 2-marker COMT haplotype had a marginally significant G × E effect on neuroticism. These results suggest that stress moderates the effects of some variants on patterns of internalizing psychopathology.

Discussion

In this study, we sought to replicate and extend previously reported candidate gene–internalizing phenotype associations in a sample of 928 out-patients with anxiety and depressive disorders. In particular, we

examined the hypothesis suggested by multivariate twin studies of the existence of both shared and disorder-specific genetic risk across internalizing disorders (genetic pleiotropy and heterogeneity). Overall, we found associations between multiple variants and primarily depression, PD/A and PTSD. The most widely associated gene was SLC6A4, although more consistently so for variants other than the 5-HTTLPR.

We replicated prior main effect SLC6A4 associations of rs6354 and rs2020936 with depression and PD/A (Wray et al. 2009). We found marginal support for previously reported main effects of SLC6A4 SNPs rs3813034 (Gyawali et al. 2010) but not rs140701 (Strug et al. 2010) on PD/A. However, we detected significant main effects for these SNPs on depression and PTSD as well as G × E effects on PD/A and PTSD. Associations were found for main effects of the short form of the 5-HTTLPR polymorphism (represented by the C-A haplotype of rs4251417 and rs2020934) with depression (significant) and neuroticism and PTSD (marginal). The current results support our prior report of association of a 4-marker high-risk haplotype of GAD1 with neuroticism and PD/A and possibly GAD (Hettema et al. 2006a). We replicated prior associations of COMT with some internalizing phenotypes but not others (Domschke et al. 2007; Pooley et al. 2007; Hettema et al. 2008). Only this gene was significantly associated with extraversion and SOC, two closely related phenotypes. A recent meta-analysis reported a small but significant effect of the rs4680 val-val genotype with neuroticism in white males (but not females), which is inconsistent with our results. In the current study, a trend for stronger association was seen for the 2-marker rs4680-rs165599 haplotype than for the val-met polymorphism rs4680 alone, supporting the need for moving beyond studies that seek to genotype only putative ‘functional’ markers. Our finding that RGS2 was not associated with any phenotype is consistent with our analysis of this gene in another sample (Hettema et al. 2013) but in conflict with some other prior reports reviewed earlier.

None of the tested variants was associated with OCD. This is consistent with a meta-analysis of 5-HTTLPR finding no association with this polymorphism (Bloch et al. 2008) but contrary to reported association with COMT val-met variant from several small studies (for a review, see Pauls, 2010); there are no prior reports for the other genetic variants tested in relation to OCD. Much debate has ensued regarding whether OCD should be considered an anxiety disorder, with overall mixed opinion based upon various categories of validators (Stein et al. 2010). The limited data from extant genetic epidemiological studies suggest that OCD does share some familial (Bienvenu et al. 2012) and genetic (Tambs et al. 2009) risk factors

with anxiety disorders, although OCD also shares risk with obsessive-compulsive spectrum disorders (Monzani *et al.* 2014). No studies have included both of these groups to determine if these represent distinct genetic factors. Overall, our results provide preliminary molecular genetic support for differentiation between OCD and other internalizing phenotypes, at least for the set of internalizing candidate genes tested herein.

We found some associations for interactive effects ($G \times E$) with recent stress in relation to the various phenotypic outcomes, with the most support for the *SLC6A4* gene. Interestingly, PTSD, which requires the etiological role of a traumatic stressor, showed these effects from four of the five polymorphisms (rs3813034, rs140701, rs6354, rs2020936), the latter two without main effects. We are aware of no prior $G \times E$ analyses using these four polymorphisms. We did not replicate prior reported $G \times E$ effects of 5-HTTLPR(S) on depression or PTSD [for recent reviews of $G \times E$ studies in these disorders, see Mandelli & Serretti (2013) and Digangi *et al.* (2013), respectively]. The only other outcome with significant $G \times E$ effects within this gene was PD/A, predicted by an interaction between stress and markers rs3813034 and rs140701, also without main effects. In addition to its main effect, we detected a marginally significant $G \times E$ interaction of the 2-marker *COMT* haplotype on neuroticism. Our findings support the hypothesis that stress moderates the effects of some genetic variants on risk for internalizing disorders. We note that the power to detect $G \times E$ effects is generally lower than for main effects of either genes or environment.

For *COMT* and *GAD1* variants, we were able to verify that genetic risk is shared between some disorder-based constructs and their predisposing dimensions of temperament, as predicted by twin studies (Hettema *et al.* 2006b; Bienvenu *et al.* 2007). For these, we tested the hypothesis that mediation explained these relationships, that is, variants had direct effects on temperament that mediated the observed effects on disorders. In particular, neuroticism mediated the effects of *COMT* and *GAD1* variants on all of their respective disorder outcomes. Both neuroticism and extraversion mediated the effects of *COMT* on depression and SOC. Although the effects of 5-HTTLPR (S) on neuroticism were only marginally significant ($p = 0.078$), we conducted an additional test to see if the mediational model of this variant on depression proposed by Munafò *et al.* (2006) was supported. Not surprisingly, that model also showed marginally significant fit to the data ($p = 0.063$). We note that interpretations pertaining to the temporal directionality of these relationships are limited by the fact that measures of temperament and emotional disorder psychopathology were obtained cross-sectionally.

We contrast our approach of using well-validated candidate genes to GWAS that take an agnostic perspective in order to discover novel risk variants. The latter have achieved great successes for many complex human traits and medical disorders and will probably play a crucial role in further elucidating the mechanisms of psychopathology. While this approach has recently provided promising findings for low prevalence, high heritability psychiatric disorders like schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014), results thus far for internalizing disorders have been less spectacular. One novel gene for PD, *TMEM132D*, has been identified via GWAS (Erhardt *et al.* 2011), while the most recent large-scale published GWAS for major depression (Ripke *et al.* 2013) and neuroticism (de Moor *et al.* 2012) have failed to yield genome-wide significant findings. More powerful samples will be needed before many well-validated risk variants for depressive and anxiety disorders can be identified via GWAS and tested for their complex role in the development of internalizing psychopathology.

Our replication of some prior gene-phenotype associations but not others, together with identification of potentially new gene-phenotype associations, can be explained in several possible ways: (1) genetic and phenotypic heterogeneity; (2) differential power to detect specific association signals (type II error); and/or (3) random (false-positive) associations (type I error), the latter two probably present in ours as well as prior studies. Additional replication studies and meta-analyses can further inform the differential likelihood of these explanations. We note that our phenotyping method, in which we constructed temperament- and diagnosis-based latent variables within patients, differs substantially from more typical case-control comparisons, suggesting another potential difference from previous studies. A potential source of discrepancy specifically for the 5-HTTLPR(s) polymorphism is the moderate coupling ($r^2 = 0.72$) of our chosen tagging 2-marker haplotype with this polymorphism (Wray *et al.* 2009); however, many prior association studies present a picture of either weak or inconsistent association overall (Willis-Owen *et al.* 2005; Risch *et al.* 2009).

In summary, we examined the potential richness and complexity of the roles of just a few candidate genes in the severity and patterning of multiple, related internalizing phenotypes in a patient population. We found possible evidence for both shared and phenotypic specific effects of some of these polymorphisms. In addition to main genetic effects, our results add to the accumulating evidence that moderation by stress and mediation by temperament provide additional mechanisms by which these genes influence susceptibility to internalizing disorders.

Supplementary material

For supplementary material accompanying this paper visit <http://dx.doi.org/10.1017/S0033291715000021>

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Declaration of Interest

None.

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