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A Large Case–Control Study of Common Functional *SLC6A4* and *BDNF* Variants in Obsessive–Compulsive Disorder

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Both serotonin transporter (*SLC6A4*) and brain-derived neurotrophic factor (*BDNF*) genes have shown positive associations with obsessive–compulsive disorder (OCD) and some other psychiatric disorders, but these results have not been consistently replicated. To explore the hypothesis that this variability might result from the effects of differing combinations of overlooked variants within *SLC6A4* together with small OCD and control sample sizes, we studied three common functional polymorphisms (5-HTTLPR, STin2, and the newly discovered SNP, rs25531) in the largest sample size of OCD patients ($N = 347$) and controls ($N = 749$) ever investigated. During methods development, we found evidence for potential *SLC6A4* genotyping problems with earlier methodology, a third possible contributor to variability in earlier studies. A fourth possible explanation might be *SLC6A4* × *BDNF* interactions, which prompted us to investigate combined genotypes of *BDNF* V66M with the three *SLC6A4* loci. Except for a nominal association with rs25531 alone, which did not survive correction for multiple comparisons, we found no evidence for any of these other variants being associated alone or together with OCD, and we therefore also examined clinical OCD subtypes within the sample to evaluate clinical heterogeneity. Subgroups based on the age of OCD onset, gender, familiality, factor analysis-derived symptom dimensions, or comorbidity with other psychiatric disorders failed to identify *SLC6A4*- or *BDNF*-associated phenotypes, with one exception of overall number of comorbid anxiety disorders being significantly associated with 5-HTTLPR/rs25531. We conclude that despite their attractiveness as candidate genes in OCD, our data provide no support for association in this large OCD patient sample and point toward the need to examine other genes as candidates for risk determinants in OCD.

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INTRODUCTION

Obsessive–compulsive disorder (OCD) is a debilitating chronic psychiatric illness with a lifetime prevalence of 2–3% of the worldwide population (Hasler *et al*, 2006; Kessler *et al*, 2005; Weissman *et al*, 1994). This disorder is characterized by recurrent and intrusive thoughts (obsessions) and repetitive behaviors (compulsions) meant to reduce distress associated with obsessions. There is a substantial body of evidence from twin and family studies indicating that OCD has a genetic component (Hettema *et al*, 2001; Rasmussen and Tsuang, 1984). Complex segregation analyses, though not entirely conclusive, point toward a complex pattern of inheritance dominated by a few genes of major effect (Hemmings and Stein, 2006). This is in keeping with results from two genome-wide scans of OCD probands which provided suggestive evidence for susceptibility loci on 9p, 3q, 7p, 1q, 15q, and 6q (Hanna *et al*, 2002; Shugart *et al*, 2006).

In addition to the above-mentioned studies, a number of candidate genes for OCD have been investigated through association analyses (Hemmings and Stein, 2006). Particular emphasis has been placed on functional variants in the serotonin transporter gene (*SERT*, *5-HTT*, *SLC6A4*), given an etiopathological hypothesis based on the effectiveness of serotonin reuptake inhibitors in the treatment of OCD. Studies have investigated common non-coding variants affecting transcriptional efficiency, such as the 43 bp promoter indel referred to as 5-HTTLPR and STin2, a variable number of tandem repeats polymorphism in intron 2 (Heils *et al*, 1995; Hranilovic *et al*, 2004; Hu *et al*, 2006; Lesch *et al*, 1995; Lovejoy *et al*, 2003; MacKenzie and Quinn, 1999). Rare coding mutations such as the gain-of-function I425V have also been reported as segregating in families with OCD probands (Ozaki *et al*, 2003). Whereas this *SLC6A4* I425V polymorphism was subsequently found in additional probands with a predominantly OCD-like phenotype (Delorme *et al*, 2005; Wendland *et al*, in press), data linking OCD with common *SLC6A4* variants have been generally inconclusive. Although a few reports found a

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positive association for the gain-of-function 5-HTTLPR L allele and OCD (Bengel *et al*, 1999; McDougle *et al*, 1998), the majority of studies have been negative (Camarena *et al*, 2001; Cavallini *et al*, 2002; Chabane *et al*, 2004; Frisch *et al*, 2000; Kinnear *et al*, 2000; Meira-Lima *et al*, 2004; Walitza *et al*, 2004). An association between OCD and the STin2 higher-expressing 12-repeat allele was reported in one study (Ohara *et al*, 1999).

Another potential explanation for the inconsistent findings seen in association studies might be a lack of appreciation of known variants. In particular, this may be relevant to 5-HTTLPR, as additional variants have been identified for both the L and S allele (Nakamura *et al*, 2000). In their recent report, Hu *et al* (2006) demonstrated that a substantial fraction of L alleles, designated L_G by the authors (which refers to the haplotype of L at 5-HTTLPR and G at dbSNP rs25531, corresponding to the originally reported allele 16-D, GenBank accession number AB031254) acts similar to the low-expressing S allele, and that only the L_A (16-A, AB031251) allele is the actual higher expressing variant. On the basis of genotyping using these functional data, a significant association between OCD and the higher expressing L_A allele and the L_AL_A genotype in both a case–control and a family-based investigation of trios was found (Hu *et al*, 2006).

One additional factor that might explain the lack of consistent findings in association analyses is the substantial clinical heterogeneity of OCD (Pato *et al*, 2002). It is thought that the diverse phenomenological and treatment presentation of this disorder may reflect, in turn, a heterogeneity of susceptibility genes. Attempts at better defining more homogeneous OCD subgroups have been made at the level of symptom presentation (Hasler *et al*, 2006), age of OCD onset (Shugart *et al*, 2006), family history (Denys *et al*, 2006), and comorbidity patterns (Leckman *et al*, 2003). Despite the theoretical plausibility of these subgroups, studies assessing candidate genes in light of more unified OCD sub-phenotypes have also been marked by inconclusive findings and a lack of replication.

The aim of this investigation was to analyze the *SLC6A4* STin2 and refined 5-HTTLPR polymorphisms in the largest OCD sample to date and in an ethnically matched control sample. In addition, we assessed the brain-derived neurotrophic factor (*BDNF*) V66M polymorphism in our sample, given the recent positive finding of its involvement in OCD (Hall *et al*, 2003). We first validated our multiplexed *SLC6A4* genotyping method (Wendland *et al*, 2006b) in light of a recent report showing magnesium chloride-dependent preferential amplification of the S allele (Yonan *et al*, 2006), and then performed a number of single locus, expression grouping, haplotype, and combined genotype analyses. Moreover, we conducted separate exploratory analyses of hypothesized OCD sub-phenotypes.

MATERIALS AND METHODS

Human Subjects

We genotyped a total of 347 OCD probands and 749 healthy control individuals. Among these, we selected 295 self-reported Caucasian adult OCD probands and 657 ethnically matched Caucasian controls for statistical comparisons. Probands were recruited through an on-going, IRB-approved adult outpatient OCD program at the NIMH Intramural Research Program in Bethesda, MD, with nationwide and international recruitment. Inclusion criteria for participation included being at least 18 years old and having a primary OCD diagnosis based on the Structured Clinical Interview for DSM-IV (SCID). Exclusion criteria included active schizophrenia or psychosis, severe mental retardation that does not permit an evaluation to characterize OCD, or OCD symptoms that occur exclusively in the context of depression (LaSalle *et al*, 2004). Peripheral blood was collected by venipuncture after written informed consent. DNA was extracted by standard procedures.

Control genomic DNA originated from three independent sources: (1) human variation panel purchased from Coriell cell repository ($N=200$ self-declared healthy US Caucasians); (2) human random control, panels 1 and 2 ($N=192$ apparently healthy, randomly selected UK Caucasian blood donors) purchased from the European Collection of Cell Cultures (Sigma-Aldrich, St Louis, MO, USA); and (3) undergraduate students ($N=357$ total, of which $N=265$ were self-declared healthy Caucasians and were used in this study) from a large Southeastern university which attracts a diverse student body from the entire US as well as $\sim$10% international students; these students participated in a separate study of genes and personality in return for partial course credit. Since none of these three control groups was clinically evaluated, we cannot rule out the possibility of individuals present in the control sample having OCD or OC symptoms, although this fraction is unlikely to be higher than the general population prevalence of 2–3% (Kessler *et al*, 2005; Weissman *et al*, 1994). Allelic and genotypic frequencies did not significantly differ between these three control groups (data not shown).

OCD Sub-Phenotypes

The current report assessed seven distinct OCD sub-phenotypes within the clinical sample. The magnitude of obsessions and compulsions was measured with the Yale-Brown Obsessive Compulsive Scale (Y-BOCS), a frequently used assessment tool for the severity of obsessive–compulsive symptoms (Goodman *et al*, 1989). The average symptom severity for the sample as a whole, as measured by the Y-BOCS, was 21.63 (SD = 9.34). We next classified the sample into either childhood or adult OCD onset groups. Consistent with the literature, those individuals with an onset younger than 18 years of age were classified as childhood onset (Samuels *et al*, 2006). The third sub-phenotype we assessed was familial history of OCD. The sample was dichotomized into either a familial group, which included those with a first-degree relative with OCD, or a non-familial group. Consistent with the literature (Cromer *et al*, in press; Hasler *et al*, 2005), we furthermore derived factor scores for the symptom dimensions of OCD using the Y-BOCS symptom checklist. Specifically, principal component analysis was applied to the 13 *a priori* Y-BOCS categories and initial factor solutions were then rotated using the Varimax procedure (Hasler *et al*, 2005; Leckman *et al*, 1997). The factor analysis generated four factor scores for each subject representing the correlation of the symptom profile of the subject with each factor. The four

factors included obsessions/checking, symmetry/ordering, contamination/cleaning, and hoarding. With regard to comorbidity features, 90% of participants were diagnosed with at least one additional lifetime Axis I psychological disorder and the number of comorbid Axis I disorders ranged from 0 to 8 (M = 2.46, SD = 1.79). Based on this information we calculated a variable for each participant representing a count of the total number of Axis I disorders, mood disorders, and anxiety disorders. The presence of past traumatic life events was assessed via diagnostic interview. Specifically, the information provided in the 'Traumatic Events List' contained in the PTSD module of the SCID was used to classify individuals into either a trauma or non-trauma group (Cromer *et al*, in press). The seventh sub-phenotype that we assessed was the presence or absence of suicidality. This information was obtained via a thorough review of the SCID and the participant's chart for evidence of suicidal ideation or suicide attempts. Statistical differences in these phenotypes per genotype were ascertained via χ^2-tests (for dichotomous phenotype definitions) and one-way analysis of variance (for continuous phenotype definitions), as appropriate. All statistical tests were performed using the Software Package for Social Sciences version 13.0.

Genotyping

The *BDNF* V66M polymorphism (dbSNP rs6265) was genotyped by 5′-exonuclease assay (TaqMan SNP genotyping assay-on-demand; Applied Biosystems, Foster City, CA, USA) using oligonucleotide primers GCCCAAGGCAGGTT CAAGAG and AACTTTCTGGTCCTCATCCAACAG as well as fluorescent probes VIC-ACTTTCGAACACgTGATAG-MGB and FAM-CTTTCGAACACaTGATAG-MGB for Val66 and Met66, respectively. In a total reaction volume of 8 μL, 5–20 ng of genomic DNA were mixed with TaqMan Universal polymerase chain reaction (PCR) master mix (Applied Biosystems) and genotyping assay to 1 × final concentrations. Thermocycling and fluorescence acquisition conditions were as recommended using an MJ Chromo4 continuous fluorescence detector (Bio-Rad, Hercules, CA, USA) connected to a PC running Opticon Monitor software version 3.1. Genotypes were scored by end point fluorescence analysis using global minimum baseline subtraction. The overall genotype completion exceeded 98%; no-template controls and randomly chosen 15% of all samples run in duplicates consistently yielded expected results.

Genotyping for *SLC6A4* 5-HTTLPR, rs25531, STin2, and I425V/I425L polymorphisms was performed by PCR/restriction enzyme digestion in duplicate for all samples: one time exactly as described previously (Wendland *et al*, 2006b), and a second time with a modified protocol scoring the loss-of-function P339L polymorphism (Glatt *et al*, 2001; Prasad *et al*, 2005) in addition to the above mentioned loci. Oligonucleotide primer sequences for P339L were CCCCTGCTGTGTTCCAGGTGTGG and CGAGGCCGTCGG TCCAATCACC; primers were diluted to 200 nM final concentration each and amplified a 211 bp large fragment (Figure 1), which was digested during the subsequent double restriction endonuclease incubation into 60 and 151 bp fragments by *Hpa*II for the Pro339 allele, but not for Leu339. Thermocycling for the expanded multiplex PCR assay consisted of 15 min initial denaturation/*Taq* polymerase activation at 95°C followed by 38 cycles of 96.5°C for 10 s and 71°C for 90 s; all other PCR and subsequent restriction enzyme digestion conditions were as described previously (Wendland *et al*, 2006b). Agreement between the genotyping results for *SLC6A4* 5-HTTLPR, rs25531, STin2, and I425V/I425L for the previously published and the expanded genotyping protocol exceeded 98%; moreover, no-template controls consistently showed no amplification. We did not observe a single undigested P339L band, supporting the notion that the Leu339 allele is extremely

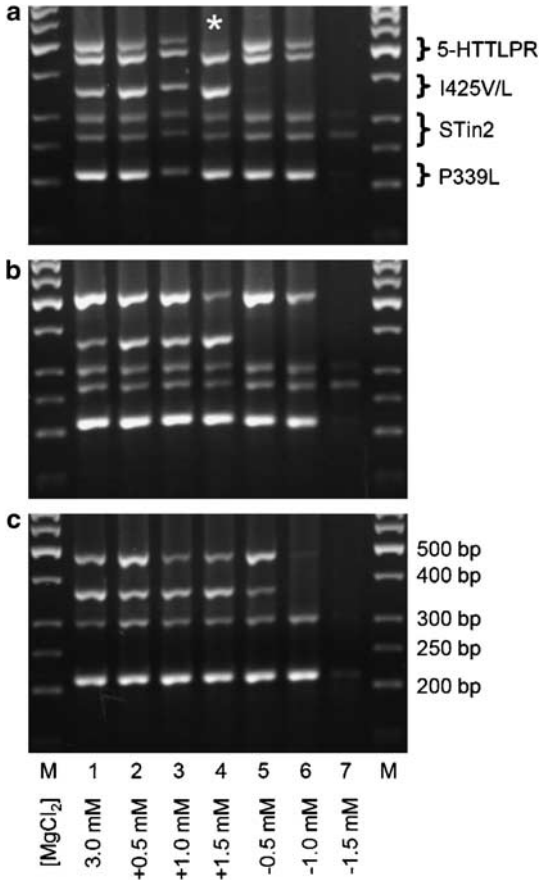

Figure 1 Magnesium chloride and dNTP titration experiments. Shown here are three representative photographs of multiplex PCR-amplified genomic DNA from (a) four 5-HTTLPR LS, (b) LL and (c) SS individuals each. Amplicon sizes for 5-HTTLPR L and S amplicons are 512 and 469 bp, respectively; 365 bp for I425 assay; 267 and 300 bp for STin2.10; and STin2.12 alleles and 211 bp for P339 assay. Seven different protocols were used: (1) no dNTPs or $MgCl_2$ added (3 mM $MgCl_2$ final total concentration with the multiplex master mix used here); (2) + 0.5 mM of $MgCl_2$ (3.5 mM final total concentration); (3) + 1.0 mM $MgCl_2$ (4.0 mM final); (4) + 1.5 mM $MgCl_2$ (4.5 mM final); (5) added 125 μM each dNTP (lowering the free $MgCl_2$ concentration by 0.5 mM as nucleotides bind MgCl2 in equimolar amounts); (6) + 250 μM each dNTP (−1.0 mM free $MgCl_2$); and (7) + 375 μM each dNTP (−1.5 mM free $MgCl_2$). Note that (a) in the SL sample and the protocol with the *highest* total $MgCl_2$ concentration of 4.5 mM (asterisk), the L band is almost completely invisible, in which case this sample might have erroneously been scored as SS. Note also that a similar effect of preferential amplification of the shorter allele for the STin2.10/12 samples (a and b) occurs with the protocol that *lowers* free $MgCl_2$ by 1.5 mM. M, 50/100 bp size markers.

rare or might even have occurred as a private mutation. Results for I425V in OCD and Coriell populations analyzed here and for 5-HTTLPR, rs25531, STin2, and I425 in the Coriell controls have been reported previously (Wendland *et al*, in press; Wendland *et al*, 2006a), data for I425V/L are not described here.

Magnesium chloride and dNTP titration experiments were carried out as follows: 20 ng of genomic DNA were mixed in a total reaction volume of 20 μL with Qiagen multiplex mix (1 × final concentration with 3 mM $MgCl_2$; Qiagen, Valencia, CA, USA) and oligonucleotide primers at previously reported concentrations for 5-HTTLPR, STin2, and Ile425 plus 200 nM of P339L primers. A total of 12 samples (four SL, LL, and SS each) were run with seven different protocols: with added $MgCl_2$ (+0.5, 1.0, and 1.5 mM added to the 3 mM $MgCl_2$ of the multiplex master mix), with added dNTPs (+125, 250 and 375 μM each; Invitrogen, Carlsbad, CA), and without supplemented $MgCl_2$ or dNTPs. All genotyping and titration experiments were carried out in hard-shell PCR plates (Bio-Rad, part number HSP9655) sealed with optical film (Bio-Rad, part number MSB1001). Two-step thermocycling was as described above, PCR products were mixed with 6 × loading dye (Fermentas, Hanover, MD, USA), separated by 3% TBE agarose gel electrophoresis at 140 V for 75 min and visualized by ethidium bromide staining. An example for each 5-HTTLPR genotype is given in Figure 1.

Statistical Analyses

Genotype data were analyzed for Hardy–Weinberg equilibrium (HWE) using the Pearson's and log likelihood ratio χ^2 as well as exact test with the de Finetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl (Cannings and Edwards, 1968). We treated 5-HTTLPR and rs25531 as two independent biallelic loci and used only 10 and 12 allele genotypes for STin2 for HWE testing. All loci did not significantly deviate from HWE either in cases or in controls with any of the three statistical tests. For the haplotype analysis of triallelic 5-HTTLPR (which our genotyping protocol scores in a phase-certain manner) and the 16 kbp downstream located STin2, phase ambiguity (double heterozygotes such as LgSa and STin2.10/12) was addressed by treating each possible haplotype as equally likely, as these two loci have been shown to not be in LD (Kim *et al*, 2002; Mynett-Johnson *et al*, 2000). Association analyses were performed using GraphPad Prism 4.03 (GraphPad software, San Diego, CA) for contingency tables with more than two columns and with Haploview 3.32 (Barrett *et al*, 2005) for 2 × 2 tables; Haploview was also used to address multiple testing bias by performing 50 000 permutations in the 'single markers only' mode with the four loci rs25531, biallelic 5-HTTLPR, STin2 (10 and 12 repeat alleles only), and *BDNF* V66M. For power calculations, we used the genetic power calculator (Purcell *et al*, 2003) under the assumption of L_A being the risk allele with 50% frequency, 2.5% disease prevalence, the genotype relative risk being 1.4 for heterozygotes, and 1.8 for homozygote risk allele carriers (based on the recent report by Hu *et al* (2006) who reported an 1.8-fold effect of the L_AL_A genotype on risk of OCD) and using 'unselected' controls. We estimate the loss of power in the permutation analyses to be at most 10% points on the basis of a recent simulation study analyzing the effects of resampling on power in case–control association studies (Rosenberg *et al*, 2006).

RESULTS

Given a recent report about $MgCl_2$-dependent preferential amplification of specific 5-HTTLPR alleles (Yonan *et al*, 2006) and the well-known overall difficulty to amplify this locus (Sutcliffe *et al*, 2005), we first performed a set of $MgCl_2$ and dNTP titration experiments to validate the robustness and reliability of our multiplex amplification of *SLC6A4*. As Figure 1 shows, the concentration of $MgCl_2$ used here for genotyping (3 mM) fell within a broad range where no preferential amplification of any 5-HTTLPR allele could be detected. At the highest $MgCl_2$ concentration, however, we observed the same phenomenon of preferential S allele amplification as Yonan *et al* (2006) recently described. Moreover, we detected a similar effect at the STin2 locus, although here the preferential amplification of the smaller amplicon (STin2.10) occurred at the *lowest* concentration (Figure 1).

We then genotyped a total of 347 OCD probands and 749 controls for three common, reported functional *SLC6A4* polymorphisms (rs25531, 5-HTTLPR, and STin2) and the *BDNF* V66M coding SNP. We present data for Caucasian probands ($N = 295$) and ethnically matched controls ($N = 657$) here, as overall numbers and diversity for non-Caucasian probands and controls provided too little power for statistical analyses. These probands comprised an independent sample not previously reported on. Our sample had more than 82% power at the $\alpha = 0.05$ level. Allelic frequencies are presented in Table 1; complete absolute and relative genotype and allele frequencies including data for males and females separately are available in Supplementary Table 2. We analyzed the classic biallelic 5-HTTLPR (S and L alleles) and rs25531 both as two independent biallelic loci and, given that our genotyping assays score both polymorphisms in a phase-certain manner, as one triallelic locus as well (S_A, L_A, and L_G; two control samples carrying an S_G allele were excluded from this analysis). Overall, only rs25531 reached a moderate level of significance when tested as single biallelic locus, including a 1.7-fold greater L_G allele ratio in OCD probands *vs* controls, but this did not survive when multiple testing bias was accounted for by permutation testing (Table 1). Moreover, we did not observe significant allelic or genotypic frequency differences between males and females nor between gender-stratified cases and controls (Supplementary Table 1).

We next analyzed whether grouping alleles and genotypes at the triallelic 5-HTTLPR locus by low and high expression (Hu *et al*, 2006) might uncover an association between 5-HTTLPR and OCD. We also performed a haplotype analysis for triallelic 5-HTTLPR and STin2. As Table 2 shows, neither of these approaches revealed a significant association between haplotypes or expression-grouped alleles or genotypes and OCD. Subsequently, combined *BDNF* and *SLC6A4* genotypes were investigated with the rationale that recent data from human (Kaufman *et al*, 2006) and mouse genetic (Ren-Patterson *et al*, 2005, 2006) studies support the

Table 1 Allelic Frequencies in Caucasian OCD Cases and Matched Controls for Common Functional Polymorphisms of *BDNF* and *SLC6A4*

		OCD probands $N = 295$	Controls $N = 657$
BDNF			
Val66Met	Val	0.807	0.808
	Met	0.193	0.192
	$\chi^2 = 0.005$, df = 1, NS		
SLC6A4			
rs25531[a]	A	0.954	0.926
	G	0.046	0.074
	$\chi^2 = 5.27$, df = 1, $P = 0.02$[a]		
Biallelic 5-HTTLPR	L	0.569	0.565
	S	0.431	0.435
	$\chi^2 = 0.03$, df = 1, NS		
Triallelic[b] 5-HTTLPR	L_A	0.524	0.493
	L_G	0.046	0.072
	S_A	0.431	0.433
	$\chi^2 = 5.23$, df = 2, NS		
STin2	9	0.012	0.024
	10	0.393	0.355
	12	0.595	0.621
	$\chi^2 = 4.84$, df = 2, NS		

[a]Uncorrected single locus contingency table analysis revealed a significant result for rs25531, but this finding became nonsignificant after multiple testing correction by permutation ($P = 0.09$).
[b]Triallelic 5-HTTLPR refers to the molecular haplotype of 5-HTTLPR and rs25531; two control samples carrying an S_G allele were omitted in this table. Complete data with absolute frequencies and gender-specific frequencies are available in Supplementary Table 1.

Table 2 Triallelic 5-HTTLPR Allelic and Genotypic Frequencies Grouped on the Basis of High and Low Expression, and Haplotype Analysis for Triallelic 5-HTTLPR and STin2

	OCD probands	Controls
5-HTTLPR expression grouping[a]		
Genotypes		
SS, SL_G, L_GL_G	0.224	0.256
SL_A, L_AL_G	0.505	0.502
L_AL_A	0.271	0.242
$\chi^2 = 1.39$, df = 2, NS		
Alleles		
S, L_G	0.477	0.507
L_A	0.524	0.493
$\chi^2 = 1.45$, df = 1, NS		
5-HTTLPR and STin2 haplotypes		
S-10	0.139	0.127
S-12	0.295	0.318
L_A-10	0.253	0.219
L_A-12	0.273	0.274
L_G-10	0.010	0.020
L_G-12	0.030	0.042
$\chi^2 = 3.12$, df = 5, NS		

[a]Reported by Hu *et al* (2006).
Two control individuals carrying one S_G allele were excluded for this analysis. There was no significant difference between probands and controls for haplotype frequencies and expression-grouped allele and genotype frequencies.

notion of functional interaction between these two genes. Combined genotype frequencies are presented in Supplementary Table 2. We did not observe significant differences between probands and controls within each combined genotype group.

Finally, we conducted exploratory analyses assessing the association between gene variants and a number of OCD sub-phenotypes. Sub-types considered included (1) OCD symptom severity, (2) childhood *vs* adult onset OCD, (3) positive familial history of OCD, (4) factor analyzed symptom dimensions, (5) total number of comorbid disorders, (6) total number of comorbid mood disorders, (7) total number of comorbid anxiety disorders, (8) suicidality, and (9) the experience of trauma. Results revealed that gene variants were associated with the total number of comorbid anxiety disorders. Specifically, the total number of comorbid anxiety disorders was significantly associated with biallelic 5HTTLPR ($F = 3.33$, $P = 0.037$), rs25531 ($F = 8.96$, $P = 0.003$), and the combined 5-HTTLPR and rs25531 genotype ($F = 3.62$, $P = 0.007$). All other analyses were nonsignificant.

DISCUSSION

The objective of the present work was to analyze common functional polymorphisms of two psychiatric disorder candidate genes, *SLC6A4* and *BDNF*, for possible involvement in OCD. Earlier investigations have evaluated the functional 5-HTTLPR polymorphism of *SLC6A4* in generally small OCD proband case–control and trio samples (and thus with low power to detect association of OCD to gene variants of small effects) with mixed results, although a recent larger study suggested that an associated promotor region SNP, rs25531, near 5-HTTLPR might have led to some of the variability (Hu *et al*, 2006). The only known functional *BDNF* variant, V66M, has been evaluated in two studies with differing results (Hall *et al*, 2003; Mossner *et al*, 2005). We addressed the lack of consistent findings by considering (1) genotyping difficulties, (2) sample size, (3) functional variants of the 5-HTTLPR L allele, (4) the

combination of *BDNF* V66M with *SLC6A4* genotypes, and (5) clinical heterogeneity. Overall, our analyses do not support the notion of a major involvement of these common functional gene variants in OCD.

Preferential amplification of specific alleles at size-polymorphic genomic loci is long known (Deka *et al*, 1992) and has recently been reported for *SLC6A4* 5-HTTLPR and STin2 (Kaiser *et al*, 2002; Yonan *et al*, 2006). Both of the latter studies have shown that the concentration of $MgCl_2$ can strongly affect genotype scoring and may thus influence the outcome of genetic analyses. This caveat prompted us to use a PCR master mix specifically optimized for size-polymorphic markers and to perform $MgCl_2$ and dNTP titration experiments, which confirmed the robustness and validity of our multiplexed assay (Figure 1). In addition to these titration experiments, we confirmed that all of our genotyped biallelic markers were in HWE. This test is frequently employed to screen for genotyping errors, and although it is neither overly sensitive nor powerful (Leal, 2005), deviation from HWE has been observed in several 5-HTTLPR genotyping studies (Greenberg *et al*, 1998; Munafo *et al*, 2005; Yonan *et al*, 2006). That being said, lack of deviation from HWE does not rule out genotyping errors, and the $MgCl_2$-dependent preferential amplification discussed above emphasizes the necessity to validate genotyping assays in future analyses of *SLC6A4* 5-HTTLPR, STin2, and other size-polymorphic loci.

We then analyzed the largest OCD sample available to date and an adequate, more than twofold larger number of matched controls for the *BDNF* V66M and *SLC6A4* STin2 and 5-HTTLPR/rs25531 polymorphisms and, overall, found no evidence of association. This proband sample constituted a new, not previously reported collection of OCD patients. Neither single locus analyses, where only the greater expressing A allele of rs25531 was moderately significantly associated with OCD and only so when multiple testing bias was not corrected for, nor haplotypes of 5-HTTLPR/rs25531 and STin2 conferred statistically significant susceptibility to, or protection against, OCD (Tables 1 and 2). Regrouping 5-HTTLPR/rs25531 alleles and genotypes on the basis of recent functional data showing functional heterogeneity of L alleles (Hu *et al*, 2006) also failed to replicate the initial positive association by these authors of the gain-of-function L_A allele and L_AL_A genotype with OCD (Table 2), even though our study was adequately powered. Superficially, it can be acknowledged that the OCD probands in our study had more L_A and fewer L_G alleles than the controls and thus were in accord with the direction of the findings of the study by Hu *et al* (2006); these differences, however, were minute and far from statistically significant. Finally, on the basis of emerging evidence from human (Kaufman *et al*, 2006) and mouse behavior genetics (Ren-Patterson *et al*, 2005, 2006) that points toward gene $\times$ gene interactions of *SLC6A4* and *BDNF*, we investigated combined genotypes for these two genes but found no support for a combined, epistatic involvement of these two genes considered together in OCD (Supplementary Table 2).

The rationales behind the analyses discussed above can be subsumed under the category of differential or interacting gene function, but they do not take into consideration an increasing awareness of phenotypical heterogeneity in OCD (Baer, 1994; Leckman *et al*, 1997; Mataix-Cols *et al*, 2002; Pato *et al*, 2002). Both categorical subtypes and dimensional approaches have been proposed to more narrowly define homogeneous populations within the larger construct of OCD (Taylor, 2005). These sub-phenotype definitions are based on an etiopathological hypothesis, which states that specific, and not necessarily overlapping, constellations of susceptibility genes, each of moderate effect size, will result in distinct phenotypes. Thus far, the overall success in genetic studies with specific OCD subtypes has been modest, which could be due to the overall small sample sizes in conjunction with the further loss of power that accompanies each stratification (Miguel *et al*, 2005).

It should also be noted that the single most strongly associated variant with OCD, *SLC6A4* I425V ('OCD 1' in OMIM), results in a clinical phenotype marked by multiple neuropsychiatric symptoms rather than a highly specific subtype, although OCD is the single most frequent diagnosis in individuals with the I425V variant (Delorme *et al*, 2005; Ozaki *et al*, 2003; Wendland *et al*, in press). Whereas initial discussions focused on the possible co-occurrence of the greater-expressing 5-HTTLPR/rs25531 alleles and related genotypes with the gain-of-function *SLC6A4* I425V variant, the presence or absence of this co-occurrence does not diminish the importance of one or another alone, especially given the nature of the different molecular mechanisms involved in their consequences for transporter function (Hu *et al*, 2006; Kilic *et al*, 2003; Lesch *et al*, 1996). We view the genetic analyses of OCD subphenotypes conducted herein as exploratory and regard the evidence for a relationship between 5-HTTLPR/rs25531 genotypes and the overall number of comorbid anxiety disorders as preliminary. More detailed analyses with larger proband samples and more specifically formulated hypotheses are required to further explore this finding.

In addition, the co-occurrence of other psychiatric disorders, and possible genes associated with them, has only uncommonly been evaluated. High comorbidity between OCD and other anxiety disorders, which was notable in an earlier study in a smaller sample of this cohort of OCD patients (LaSalle *et al*, 2004), was found in the present study to be significantly associated with both *SLC6A4* promotor–region variants, alone and together, with the strongest statistical association being driven by rs25531. Since these two variants interact functionally, this raises the question of whether previous observations of variability in associations between OCD and 5-HTTLPR alone might have been contributed to by different frequencies of comorbid disorders in the diverse OCD populations investigated. As rs25531 has only been previously evaluated in the single study by Hu *et al* (2006), which did not consider the question of comorbidity, it might be that rs25531 covertly influenced OCD-5-HTTLPR associations in earlier studies. Likewise, with regard to a possible *SLC6A4-BDNF* interaction in OCD, comorbidity in particular with major depressive disorder, might also help explain the lack of replication in our study with the previous strong finding of a protective effect of the *BDNF* Met66 allele in OCD by Hall *et al* (2003). TheV66M polymorphism has been reported in several studies as significantly associated with different depressive disorders (Angelucci *et al*, 2005), and well as

with schizophrenia (Neves-Pereira *et al*, 2005), and anorexia nervosa (Ribases *et al*, 2004).

The case–control association study design employed here is generally susceptible to population stratification. Although it has greater statistical power than family studies, its results can be spurious for loci that differ in their allelic frequencies between ethnicities. The 5-HTTLPR polymorphism is known to vary strongly between ethnicities (Gelernter *et al*, 1999; Greenberg *et al*, 2000; Kunugi *et al*, 1997), and we addressed this issue by only analyzing ethnically matched probands and three control groups. Our three control groups were derived from three different geographical locations, and we thus cannot completely rule out some degree of remaining hidden population stratification (despite lack of differences in allelic and genotypic frequencies among these samples), given the recent report by Hu *et al* (2006) that showed marked differences in allelic frequencies at the 5-HTTLPR locus between Caucasian populations, even though these were partially comprised of individuals with psychiatric diagnoses. It should also be noted that the recent association between 5-HTTLPR and OCD by Hu *et al* was observed in a case–control as well as in a family-based association study, the latter of which is not susceptible to hidden population stratification. Thus, the lack of association in our study warrants further analyses in large family-based OCD samples such as the OCD Collaborative Genetics Study (Samuels *et al*, 2006) before it will point toward the need to analyze other candidate regions such as 9p, 3q, 7p, 1q, 15q, 6q, and 13q (Hanna *et al*, 2002; Shugart *et al*, 2006; Willour *et al*, 2004) and polymorphisms involved in other neurotransmitters such as glutamate (Arnold *et al*, 2006; Dickel *et al*, 2006; Rosenberg and Keshavan, 1998), neuromodulators, and developmental signals potentially of importance in OCD such as glutamate.

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