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Tryptophan Hydroxylase-1 Gene Variants Associate with a Group of Suicidal Borderline Women

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Alterations in the serotonin (5-HT) system have been related to impulsive aggression and suicidal behavior, common features of the borderline personality disorder (BPD). Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in 5-HT biosynthesis. Two isoforms are known, TPH-1 and TPH-2. *TPH-1* has been correlated to various psychiatric and behavioral disorders by gene polymorphism association studies. We aimed to determine whether specific *TPH-1* haplotypes associate with BPD. A case–control design was employed. The control group included 98 women without psychiatric history. In all, 95 patients were included, all Caucasian women with a BPD diagnosis who had attempted suicide at least twice during their lifetime. Exclusion criteria were: (i) substance dependence; (ii) dementia or other irreversible organic brain syndromes; (iii) psychotic disorders or major depressive illness with melancholic features; (iv) life-threatening eating disorders. Six single-nucleotide polymorphisms (SNPs) were found at significant linkage disequilibrium across 23 kb of the *TPH-1* gene in both patients and controls, suggesting a haplotype block structure. While no individual SNP showed association, several haplotypes associated with the BPD group. In particular, one six-SNP haplotype was absent from the control group while representing about one-quarter of all haplotypes in the BPD group (corrected $P \ll 10^{-5}$). A 'sliding window' analysis attributed the strongest disease association to haplotype configurations located between the gene promoter and intron 3. We conclude that *TPH-1* associates with BPD in suicidal women. Our data support the expectation that haplotype analysis is superior to single locus analysis in gene–disease, case–control association studies.
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INTRODUCTION

Borderline personality disorder (BPD) is a serious mental condition affecting 1–2% of the general population (Swartz *et al*, 1990). BPD psychopathology is related to contributing elements such as interpersonal stress, affective instability, impulsivity, dissociation, and self-injurious behavior (Spitzer *et al*, 1992). Such elements are proposed to play a key role in the development of the disorder, and may be expressed to various degrees in different patients with BPD (Lieb *et al*, 2004). The disease often disrupts family and work life, long-term planning, and the individual's sense of identity.

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Several epidemiological studies indicate a predominance of BPD in women (about 70 *vs* 30% men) (Swartz *et al*, 1990). BPD is associated with a history of physical or sexual trauma during childhood, which is reported by 40–70% of in-patients. The severity of borderline psychopathology has also been linked to severity of childhood sexual abuse (Silk *et al*, 1995; Zanarini *et al*, 2002). Behaviorally, BPD patients show a high rate of self-injury without suicide intent, as well as a high rate of suicide attempts and completed suicide in severe cases (Soloff *et al*, 1994). About 40–80% of patients with BPD report a history of major depression, and often meet Diagnostic and Statistical Manual (DSM) criteria for other psychiatric disorders such as substance abuse, post-traumatic stress disorder (PTSD), anxiety disorders, and eating disorders (Lieb *et al*, 2004).

Several environmental and genetic factors are considered predisposing elements (Siever *et al*, 2002; Skodol *et al*, 2002; Lieb *et al*, 2004). Environmental factors such as sexual abuse or situations of abandonment are proposed to induce dysfunctional behaviors and psychosocial conflicts, which in turn might cause emotional dysregulation and

impulsivity (Lieb *et al*, 2004). A genetic predisposition to impulsivity and harm avoidance might contribute to an individual's vulnerability to the disorder (Siever *et al*, 2002). The role of a predisposing genetic background has been investigated in twin studies (Skodol *et al*, 2002). Torgersen (1984, 1987) and Torgersen *et al* (2000) have presented several twin studies over the years. Earlier studies from this group on a small sample of twins indicated that environment is important for development of BPD, while genetics is not (Torgersen, 1984). In another, more recent and larger study concordance rates of 35% in monozygotic (MZ) and 7% in dizygotic (DZ) twin pairs were observed, suggesting a strong genetic effect in the development of the disorder (Torgersen *et al*, 2000). However, more studies are required to estimate the contribution of genetic and environmental elements.

Impulsive aggression and affective instability are key domains among the contributing elements of BPD (Skodol *et al*, 2002). Affective instability has been associated with increased responsiveness of the cholinergic system, while impulsive aggression correlates inversely with serotonergic activity in the brain (Skodol *et al*, 2002). Serotonin (5-HT) neurotransmission is related to anxiety in animal models as well as in human (Cloninger, 1987; Griebel, 1995). 5-HT turn-over is particularly active in cortical and limbic areas involved in emotional aspects of behavior (Whitaker-Azmitia *et al*, 1990; Westenberg, 1996). There is extensive evidence for the presence of 5-HT nerve terminals and/or 5-HT receptors in neuroendocrine regions such as hippocampus, hypothalamus, and brainstem (Chaouloff, 1993). Clinically, reduced 5-HT uptake has been associated with depression and anxiety (Iny *et al*, 1994; Owens and Nemeroff, 1994). The notion that the 5-HT system plays an important role in impulsive behavioral patterns is based on various studies. Structural and functional neuroimaging data show dysfunctional areas in brain networks, such as the anterior cingulated cortex (ACC), the hippocampus, and the amygdala, which are also involved in dysfunctional 5-HT neurotransmission (Soloff *et al*, 2003). Other evidence stems from studies regarding impulsive aggression, violent behaviors, and various suicidal behaviors in correlation with 5-HT metabolites such as 5-hydroxyindole acetic acid (5-HIAA) (Cochran *et al*, 1976; Linnoila *et al*, 1983; Brown *et al*, 1989). Decreased levels of 5-HIAA have been observed in self-directed aggression such as violent suicidal behavior (Asberg *et al*, 1976), and also in externally directed aggression (Linnoila *et al*, 1983; Brown *et al*, 1989). Post-mortem studies of suicidal victims have also revealed reduced level of brain 5-HT (Cochran *et al*, 1976; Korpi *et al*, 1986). Although the 5-HT system has been implicated in the etiology of impulsive aggression, the exact nature of the dysfunction remains unsolved.

Impulsive aggression is a liability component in the heritability of suicide and suicidal behavior (Brent and Mann, 2005). Twin studies have shown an increased suicide risk among co-twins even after controlling for other risk factors (Statham *et al*, 1998; Fu *et al*, 2002). In a large Australian twin study, genetic factors accounted for 45% of the variance in suicide thoughts and behavior (Statham *et al*, 1998). Several twin and adoption studies indicate that suicidal behavior is heritable, and heritability includes both suicide attempts and suicide completion (Brent and Mann, 2005). Both completed suicide and suicidal behavior have been reported to have higher concordance rates in MZ than in DZ twins (14.9 *vs* 0.7% and 23 *vs* 0.7%, respectively) (Roy and Segal, 2001). Some overlap between heritability of suicidal ideation and actual suicidal behavior has been noted in twin studies (Fu *et al*, 2002).

One of the 5-HT system genes that have been studied in relationship to aggression, anger, impulsivity, and suicidal behavior is tryptophan hydroxylase (*TPH*) (Abbar *et al*, 1992; Mann *et al*, 1997; Nielsen *et al*, 1998; Kunugi *et al*, 1999). Since TPH is the rate-limiting enzyme in the biosynthesis of 5-HT (Cooper and Melcer, 1961), *TPH* variants might be expected to be related to pathogenesis events involving dysfunction of the 5-HT system. As such, *TPH* is potentially one of the major candidate genes for genetic predisposition to BPD, particularly in patients with suicidal behavior (Roy *et al*, 1997). Until recently, only one gene encoding TPH was known, which we refer to as *TPH-1*. The gene is located in the human chromosome 11p15.3–p14, is about 29 kb long, and includes 11 exons (Craig *et al*, 1991). Recently, a second TPH isoform has been reported, thereby called TPH-2 (Walther and Bader, 2003). Its gene is located on chromosome 12 and shows 71% homology to *TPH-1* on the amino-acid level (Walther and Bader, 2003). *TPH-1* and *TPH-2* are expressed in nearly equal amounts in several human brain regions such as frontal cortex, thalamus, hippocampus, hypothalamus, and amygdala. *TPH-2* is predominantly expressed in the brain stem whereas it is absent from peripheral tissues such as heart, lung, kidney, duodenum, and adrenal gland, where only TPH-1 is expressed (Zill *et al*, 2004a). The two most widely investigated single nucleotide polymorphisms (SNPs) in *TPH-1* are A218C (rs1800532) and A779C (rs1799913) (Tsai *et al*, 1999). Significant associations have been reported between SNP A779C and cerebrospinal fluid (CSF) 5-HIAA concentrations among Finnish impulsive offenders (Nielsen *et al*, 1994). The polymorphism was also associated with violent offenders who had a history of suicide attempts (Nielsen *et al*, 1997). Furthermore, associations were observed in a group of depressed alcoholics (Mann *et al*, 1997). However, while allele 799C was associated with the mentioned Finnish patients, in the latter study allele 799A was more represented in depressed suicide attempters (Mann *et al*, 1997; Nielsen *et al*, 1998). In an extensive study, seven *TPH-1* polymorphisms were investigated in relationship to suicidal behavior. The strongest associations were found for violent suicidal attempters with no history of depression (Abbar *et al*, 2001).

While the studies cited above are likely to point all to the same direction, results are conflicting, as it is often the case for single marker analyses in case–control studies, which seem prone to inconsistency in common disease association studies (Cardon and Bell, 2001; Lohmueller *et al*, 2003). This is generally attributed to the fact that common psychiatric disorders are expected to associate with common alleles. Thus, the use of haplotypes as more specific risk markers than single alleles is currently being explored (Judson *et al*, 2000; Clark, 2004). Alleles are mostly organized in haplotype blocks, that is, limited arrays of allelic combinations inherited with minimal recombination, and rather stably maintained in populations (Gabriel *et al*, 2002). However, reliable construction of such blocks may require

parent–offspring transmission data, which are often not available. An alternative concept directed toward molecular pathogenic studies is the gene-based haplotype, that is, a combination of alleles located within a gene unit, independently of genetic heritability (Hoehe, 2003). Gene-based haplotypes do not necessarily correspond to haplotype blocks, as individual haplotype blocks may contain more than one gene, and one gene unit may span over several haplotype blocks. Potentially, detailed gene-based haplotypes could be the most precise markers possible for a given gene, as they would contain all the variations in the gene (Hoehe, 2003).

We have initiated studies aimed to apply the use of gene-based haplotypes as a starting point for the identification of risk haplotypes within a classical case–control study design. We recently found six SNPs within TPH-1, spanning over 23 kb of the total 29 kb, all at significant linkage disequilibrium (LD) with each other in a group of healthy individuals (Gizatullin *et al*, 2005). The SNPs are likely to belong to the same haplotype block. Here, we report a risk haplotype analysis based on these SNPs, carried out on a group of female BPD patients.

MATERIALS AND METHODS

Human Subjects

This study was approved by the Ethics Committee of the Karolinska Hospital. All subjects were unrelated Caucasian women living in the Stockholm County. Cases and controls genotyped in this study were matched for gender, ethnicity, and geographical distribution. Subjects were interviewed using the Structured Clinical Interview for DSM-III-R (Spitzer *et al*, 1990) or DSM-IV (Spitzer *et al*, 1992). The control group included 98 female individuals (mean age $\pm$ standard deviation: 44.0 $\pm$ 13.8 years) recruited as reported earlier (Gustavsson *et al*, 1999). Briefly, subjects were either re-examined healthy individuals, mainly staff and medical students, or subjects drawn from the general population for previous biological psychiatric studies performed at the Karolinska Institute. At the time of blood collection and diagnostic interview, none of the individuals in this group had any psychiatric history. For the recruitment of the BPD group, information regarding a dialectic behavioral therapy (DBT) project was distributed to all Stockholm County Council psychiatric clinics, serving a population of approximately 1.8 million inhabitants. These clinics referred 162 patients, all women, with a DSM-IV BPD diagnosis. One inclusion criterion for recruitment of patients in this study was at least two potentially life-threatening suicide attempts, with at least one attempt made less than 6 months prior to referral. Lethal intent was defined according to the patient's belief that the attempt could have been lethal. Exclusion criteria were: (i) a current diagnosis of substance dependence; (ii) evidence of dementia or other irreversible organic brain syndrome; (iii) a current diagnosis of a psychotic disorder or major depressive illness with melancholic features; (iv) a life-threatening eating disorder. A total of 95 patients (mean age $\pm$ standard deviation: 30.5 $\pm$ 8.1 years) were included in the present study.

The latest suicide attempt before study inclusion was carried out with the following methods: drug overdose (70.5%), penetrating lesions (21%), hanging or strangulation (6.3%), jumping (1.1%), poisoning (1.1%). Most patients also met DSM-IV criteria for a diagnosis of major depression or anxiety disorders. Specifically, 68 patients (72%) had a diagnosis of major depression, 62 (65%) a diagnosis of an anxiety disorder, and 37 (39%) had both diagnoses.

Genotyping

Venous blood was drawn and immediately frozen in aliquots at -70°C or below until analyzed. Genomic DNA was prepared from whole blood by using QIAamp® DNA Blood Mini kit (Qiagen, CA, USA). The extracted DNA was stored at 4°C until analyzed. DNA (50 ng/reaction) was amplified by polymerase chain reaction (PCR), carried out in a T3 Thermocycler (Biometra® GmbH) in a total volume of 25 µl. The reaction buffer was composed of 1.5–2.5 mM $MgCl_2$, 67 mM Tris-HCl pH 9.2, 16.6 mM $(NH_4)_2SO_4$, 0.1% v/v Tween 20, 200 µM dNTPs, 20 pmol of each primers (MWG Biotech AG), and 0.75 U of *Taq* DNA polymerase (Roche Diagnostic GmbH). The PCR products were then digested overnight with appropriate restriction enzymes (see Table 1), subjected to electrophoresis on 2% agarose gels (Roche Diagnostic GmbH), and visualized after an ethidium bromide staining. Table 1 shows primer sequences, PCR annealing temperatures, and restriction enzymes used for each SNP.

SNP rs1799913 was analyzed by pyrosequencing, using a Pyrosequencer PSQ 96 and a PSQ 96 SNP Reagent Kit (Pyrosequencer, Uppsala, Sweden) according to the manufacturer's instructions. PCR reactions were carried out in 50 µl volume containing 100 ng genomic DNA, PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% v/v Tween 20), 200 µM dNTPs, 10 pmol of each primers (the reverse primer was biotinylated), and 1.5 U of *Taq* DNA polymerase (Roche Diagnostic GmbH). Thermal cycling was performed with an initial denaturation for 5 min at 96°C, followed by 50 cycles of denaturation for 30 s at 96°C, primer annealing for 30 s at 55°C, and synthesis for 30 s at 72°C. A final primer extension was conducted for 5 min at 72°C. The PCR products (140 bp) were run on 2% agarose gels (Roche Diagnostic GmbH), and visualized after an ethidium bromide staining. A total 45 µl of PCR product were used for pyrosequencing, and 15 pmol of the forward sequencing primer were applied to detect the polymorphisms.

Statistical Analyses

Genotype and allele frequencies, as well as Hardy–Weinberg equilibrium were calculated using Microsoft Excel macro PHARE version 2.1 (developed by David Cox), which can be downloaded at http://bioinformatics.org/macroshack/programs/PHARE/description.html.

For association analyses of individual genotypes and alleles, χ^2 analysis on 2×2 or 2×3 contingency tables was carried out (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html). The significance level for all statistical tests was 0.05.

Table 1 Primers for PCR Amplification and Pyrosequencing

Marker	SNP ID[a]	SNP position[b]	Primer sequence	Annealing temperature	Restriction enzyme	Primer position[b]
1	rs4537731	124 889	TTTATGGCATTGAAGTAAGAGCAC	55°C	*Sau*IIIA	125 089–125 066
			TTTTGGCTCCTGGCACTTAAC			124 772–124 792
2	rs684302	116 360	AGAGAGATGGAGCAAAACACTAC	55°C	*Alu*I	116 248–116 270
			CCAGTCCTTCCAAATCTGATAC			116 459–116 438
3	rs211105	111 308	CAAGGCAAGATTTATATGAGTT	55°C	*Cfr*I	111 050–111 071
			CTCAGGAAAACAGAAGGGTAGGGT			111 481–111 458
4	rs1800532	103 821	AATGGCATCTACCTTATGGGTTC	58°C	*Nhe*I	103 993–103 971
			CTTTATTTTTCTCCATGGGACTCA			103 655–103 678
5	rs1799913	103 260	ATTGGATTTCGATTTGATTG	55°C	*Pyr.*[c]	103 335–103 316
			GGCAAAACTAGGTTCAGC			103 196–103 213
			CAGCGTGACAAACTTGTACC			103 283–103 264
6	rs7933505	101 992	CCCCAAAGCTTTTGTTGTGCGT	55°C	*Bau*I	101 746–101 767
			TCAGATTCCACATTGCCGTTGAAC			102 194–102 171

[a]SNP ID number from the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/).
[b]SNP and primer positions are shown based on NCBI clone AC124058.
[c]For detection of SNP rs1799913, pyrosequencing was used.

Table 2 Allele Distribution in the Study Population

		Allelic frequencies		
SNP[a]	Polymorphism[b]	Control ($n=98$)	BPD ($n=95$)	*P*-value[c]
1	A/G	0.48/0.52	0.45/0.55	0.462
2	C/T	0.52/0.48	0.63/0.37	**0.035**
3	G/T	0.21/0.79	0.27/0.73	0.172
4	C/A	0.55/0.45	0.61/0.39	0.164
5	C/A	0.56/0.44	0.64/0.36	0.053
6	G/A	0.46/0.54	0.60/0.40	0.137

Bold numerals highlight significant SNPs (determined by a significant *P*-value).
[a]SNP numbering as in Table 1.
[b]Allele1/allele2.
[c]Not corrected for multiple testing.

For pair wise LD and haplotype analyses, the Arlequin program version 2.0 was used (http://lgb.unigene.ch/arlequin). A total of 100 000 permutations were performed in each analysis. Lewontin's D'-values were used to illustrate the extent of LD, and the corresponding *P*-values are also shown. Bonferroni correction was used for multiple testing.

RESULTS

Single Locus Analysis

Subjects were genotyped for the SNPs indicated as #1–6 in Table 1. All SNPs were at Hardy–Weinberg equilibrium in both patient and control groups. Results of single locus association analyses are summarized in Tables 2 and 3. No genotype association was found with the disease. Allele association tests for BPD *vs* controls showed significance for SNP 2 C allele with BPD ($P=0.035$; Table 2). However, this association did not maintain statistical significance after Bonferroni correction.

LD Analysis

LD data indicated as Lewontin's D'-values and corresponding *P*-values were calculated for all SNP pairs in control and BPD groups as shown in Tables 4 and 5. A comparison of BPD patients to controls shows that both groups shared an almost homogeneous LD pattern with the exception of SNP 1 in the control group. This SNP was found to be in strong LD only with SNP 3. As for the other SNPs in this group, all show significant LD with *P*-values $<10^{-5}$. All SNPs were in high LD with each other in the patient group, with *P*-values $<10^{-5}$.

Six-Marker Haplotype Analysis

Our ultimate goal is to characterize the entire sequence of risk gene variants. To this end, haplotype-based gene variants serve as a basis to a complete sequence characterization. Six-marker haplotype analyses were carried out across the *TPH-1* gene for the entire study population. Table 6 shows the estimated haplotype frequencies with Bonferroni corrected *P*-values. About 80% of the healthy group carried six common haplotypes (#1–6 in Table 6), all with frequencies above 7%. Significant differences were observed between groups.

Haplotypes 3 (GTTAAA) and 5 (GCTCCG) were significantly associated with BPD, each having a frequency almost three-fold higher in BPD patients than in healthy subjects ($\chi^2=22.5$, df = 1, corrected $P<10^{-5}$, and $\chi^2=10.1$, df = 1, corrected $P=0.0084$, respectively). Haplotypes 1, 2, and 4 showed an opposite pattern, as they were associated with the healthy control group (Table 6). Haplotype 1 (ATTAAA) was carried by 26.6% of the controls *vs* 5% of the patients ($\chi^2=32.4$, df = 1, corrected $P<10^{-5}$). Haplotypes 2 (GCGCCG) and 4 (GTGCCG) differ only in their

Table 3 Genotype Distribution in the Study Population

		Genotypic frequencies (%)						
		Control			BPD			
SNP[a]	Polymorphism[b]	1–1	1–2	2–2	1–1	1–2	2–2	*P*-value[c]
1	A/G	27.6	41.8	30.6	22.1	45.3	32.6	0.681
2	C/T	33.7	37.8	28.6	41.1	44.2	14.7	0.066
3	G/T	3.1	35.7	61.2	6.3	41.1	52.6	0.355
4	C/A	29.6	51.0	19.4	48.9	46.3	14.7	0.356
5	C/A	29.6	52.0	18.4	45.3	40.0	14.7	0.079
6	G/A	28.6	50.0	21.4	38.9	44.2	16.8	0.300

[a]SNP numbering as in Table 1.
[b]Allele1/allele2.
[c]Not corrected for multiple testing.

Table 4 *D′*- and *P*-Values for *TPH-1* SNP Combinations in Healthy Controls[a]

SNP[b]	1	2	3	4	5	6
1		$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
2	0.467		$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
3	**0.771**	**0.785**		$<10^{-5}$	$<10^{-5}$	0.0008
4	0.453	**0.766**	**0.815**		$<10^{-5}$	$<10^{-5}$
5	0.433	**0.738**	**0.823**	**0.754**		$<10^{-5}$
6	0.462	**0.824**	**0.664**	**0.866**	**0.816**	

[a]Upper diagonal: *P*-values for pair wise LD; lower diagonal: *D′*-values for each SNP pair combination.
[b]SNP numbering, as listed in Table 1, follows the physical location on the gene. *D′*-values >0.5 are shown in bold.

Table 5 *D′*- and *P*-Values for *TPH-1* SNP Combinations in BPD Patients[a]

SNP[b]	1	2	3	4	5	6
1		$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
2	**0.606**		$<10^{-5}$	$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
3	**0.779**	**1**		$<10^{-5}$	$<10^{-5}$	$<10^{-5}$
4	**0.636**	**0.700**	**1**		$<10^{-5}$	$<10^{-5}$
5	**0.628**	**0.686**	**1**	**1**		$<10^{-5}$
6	**0.563**	**0.646**	**1**	**1**	**1**	

[a]Upper diagonal: *P*-values for pair wise LD; lower diagonal: *D′*-values for each SNP pair combination.
[b]SNP numbering, as listed in Table 1, follows the physical location on the gene. *D′*-values >0.5 are shown in bold.

second SNP (rs 684302, C/T), and both maintained significance after correction for multiple testing ($\chi^2 = 23.1$, df = 1, corrected $P < 10^{-5}$, $\chi^2 = 18.3$, df = 1, corrected $P = 0.0001$, respectively). All rare haplotypes with frequencies below 3% in both groups were pooled, since an individual haplotype analysis would have limited statistical power. This compound group was associated with the healthy control group (22.4 *vs* 7.4% in patients, $\chi^2 = 17.2$, df = 1, corrected $P = 0.0002$).

Table 6 Estimated Haplotype Frequencies

		Frequencies (%)			
	Haplotypes[a]	Control	BPD	χ^2	*P*[b]
1	**A T T A A A**	26.6	5.0	32.4	$<10^{-5}$
2	**G C G C C G**	16.4	1.9	23.1	$<10^{-5}$
3	**G T T A A A**	9.4	28.1	22.5	$<10^{-5}$
4	**G T G C C G**	9.1	0.0	18.3	0.0001
5	**G C T C C G**	8.4	19.9	10.1	0.0084
6	**A C T C C G**	7.7	12.7	2.6	0.6228
7	**A C G C C G**	0.0	24.9	55.2	$<10^{-5}$
8[c]	**All rare <3%**	22.4	7.4	17.2	0.0002

[a]Alleles are ordered according to their physical location on the gene, as listed in Table 1.
[b]*P*-value calculated for each haplotype vs all others by the χ^2 test and corrected by Bonferroni method.
[c]All haplotypes with estimate frequencies <3% in both groups.
Significant *P*-values are shown in bold.

A striking observation was made for haplotype 7 (Table 6, ACGCCG). This haplotype was absent in the control group, while it displayed a 24.9% frequency in the BPD group ($\chi^2 = 55.2$, df = 1, corrected $P \ll 10^{-5}$). Except for the first SNP (rs4537731, A/G), all alleles of this haplotype are opposite to those in haplotype 1 (ATTAAA), which was associated with the control group.

Three-Locus Haplotype 'Sliding Window' Analysis

The presence of a causal polymorphism within a gene region may be reflected in a particularly strong association with disease for that region. We therefore analyzed haplotypes formed by three-locus combinations with a 'sliding window' approach, that is, SNP combinations 1–2–3, 2–3–4, 3–4–5, and 4–5–6. Table 7 shows the three-locus

Table 7 Three-Locus Haplotype Analysis[a]

Configurations[b]	Three-locus haplotypes	Frequencies (%)		χ^2	P^c
		Control	BPD		
1–2–3	ACG	0.49	23.6	49.1	$<10^{-5}$
	ATT	33.8	5.8	47.9	$<10^{-5}$
	GCG	18.8	1.8	30.4	$<10^{-5}$
	GCT	11.5	23.4	10.4	0.007
	GTG	9.1	1.0	13.8	0.001
	GTT	12.1	29.7	17.8	0.0001
2–3–4	CTC	19.9	34.5	10.6	0.007
	TGC	10.1	1.0	15.9	0.0006
	TTC	6.8	1.0	8.6	0.020
3–4–5	TCA	4.8	0.0	10.5	0.007
	TCC	21.7	35.5	8.9	0.017
4–5–6	CAG	4.1	0.0	8.3	0.023
	CCG	46.4	61.0	8.4	0.022

[a]Only haplotypes with significant associations are shown.
[b]Totally, four configurations are made by the six SNPs. Each configuration contains eight different combinations of three-locus haplotypes ($4 \times 8 = 32$). Numbers in each configuration indicate which SNPs are included. The position of each SNP is according to their physical location on the gene, as listed in Table 1.
[c]*P*-values after Bonferroni correction.

haplotypes that carried significant associations after Bonferroni correction. Most of the significant associations mapped in the configuration 1–2–3, encompassing the gene region comprised between promoter and intron 3. Haplotype combinations ACG, GCT, and GTT in this configuration were associated with the patient group (corrected $P \ll 10^{-5}$, $P = 0.007$, and $P = 0.0001$, respectively), while haplotypes ATT, GCG, and GTG were associated with the control group (corrected $P \ll 10^{-5}$, $P \ll 10^{-5}$, and $P = 0.0012$, respectively). Configuration 2–3–4 carried three significant combinations, namely haplotype CTC was associated with BPD (corrected $P = 0.007$), while haplotypes TGC and TTC were associated with healthy controls (corrected $P = 0.0006$ and $P = 0.020$, respectively). Configurations 3–4–5 and 4–5–6 each provided two significant haplotype combinations. In particular, haplotype TCC (configurations 3–4–5) was among the most frequent haplotypes (35.5% in patients *vs* 21.7% in controls, corrected $P = 0.017$). The most common three-locus haplotype among those tested was CCG (configuration 4–5–6), with frequencies of 61% in patients *vs* 46% in controls (corrected $P = 0.022$).

DISCUSSION

We have identified several *TPH-1* gene variants associated with BPD. *TPH-1* has been associated with a number of psychiatric disorders including mood disorders, violent behavior, and suicide (Mann *et al*, 1997; Skodol *et al*, 2002; Arango *et al*, 2003). Most reports on *TPH-1* have been association studies with individual markers (Arango *et al*, 2003). We are not aware of any *TPH-1* association study using SNP-based haplotypes in the context of BPD.

The notion that the 5-HT system is implicated in violent and suicidal behavior is among the best established in biological psychiatry (Asberg *et al*, 1976; Skodol *et al*, 2002; Arango *et al*, 2003; Lieb *et al*, 2004). However, associating a group defined by diagnosis rather than behavior to specific biological findings has proven less amenable to experimentation (Leboyer *et al*, 1998). This is possibly due to a number of factors, related among others to both genotype and phenotype stratification. In the present studies, we have attempted to restrict both sources of variability, by (i) focusing on a patient group sharing ethnicity, gender, a specific DSM IV (Spitzer *et al*, 1992) diagnosis, and suicidal behavior; and (ii) analyzing genetic susceptibility by way of a specific gene-based haplotype analysis approach. However, as mentioned in the introduction, we have recently initiated studies aimed at applying the concept of gene-based haplotype to the identification of risk haplotypes within a classical case–control study design. The six SNPs analyzed here were chosen because of a homogeneous LD pattern previously observed in a group of about 450 individuals (Gizatullin *et al*, 2005). In the present study, LD analysis showed that both groups share a homogeneous LD pattern with the exception of SNP 1 in the control group, which was in strong LD only with SNP 3. All other SNPs in the control group, as well as all SNPs in the BPD group were in strong LD with each other. The results support our initial observations, and suggest that all the SNPs belong to a single haplotype block. Therefore, it becomes of interest to see how such a set of SNPs behave when haplotypes are treated as risk markers.

Analyses of individual SNPs showed a marginal association with BPD for SNP 2 C allele ($P = 0.035$) with BPD (Table 2). However, no genotype association was observed, and the allele association did not maintain statistical significance after Bonferroni correction. Single marker association studies are known to be prone to generate weak associations and/or inconsistent reproducibility (Cardon and Bell, 2001; Lohmueller *et al*, 2003). This is due to several reasons, one of which being the fact that parent–offspring transmission of genetic variants follows certain constraints, such that most individual alleles carry only limited information on gene variants (Clark, 2004). Alleles are mostly organized in haplotype blocks, limited arrays of allelic combinations inherited with minimal recombination, and rather stably maintained in populations (Gabriel *et al*, 2002).

When all six SNPs were included in a haplotype analysis, almost 80% of the healthy group carried six common haplotypes, all with frequencies above 7%. However, significant differences were observed between groups. The most striking observation was made for haplotype ACGCCG, which displayed a 25% frequency in the BPD group while being completely absent in the control group (corrected $P \ll 10^{-5}$). We had previously carried out the same haplotype analysis in a group of 166 major depression female outpatients with about 50% comorbidity for anxiety disorders but no history of personality disorders. In that patient group, haplotype ACGCCG was almost absent

(0.25% frequency, unpublished data), which suggests that the observed association is likely specific for BPD and/or suicidal behavior.

Haplotypes GTTAAA and GCTCCG were also significantly associated with BPD, each having frequencies almost three-fold higher in BPD patients than in healthy subjects. Among common haplotypes with a possible protective role, ATTAAA was carried by 26% of the controls *vs* 5% in the BPD group, while GCGCCG had a frequency of about 16% in controls *vs* 2% in the BPD group. It is interesting to note that the opposite haplotypes TTAAA and CGCCG (configuration 2–3–4–5–6) were associated with either group depending on SNP 1 allelic phase, that is, the presence of SNP 1 alleles A or G seemed to determine whether the resulting six-loci haplotype was associated with risk or protection. However, SNP 1 alone was not significantly associated with either group, indicating that its role is probably context-specific. In agreement with this observation, the *TPH-1* gene region that carried the strongest association with BPD mapped in the SNP configuration 1–2–3, encompassing the gene region comprised between the promoter and intron 3. Three haplotype combinations in this configuration associated strongly with the disorder, while three more haplotypes associated strongly with the control group. The *TPH-1* promoter region polymorphism has been analyzed in detail (Rotondo *et al*, 1999), and it appears to be characterized by very few haplotypes, since SNPs are almost invariably in complete LD resulting in two major haplotype blocks. The implication is that SNP 1 alleles might be considered representative of the entire promoter region sequence (approx. 2 kb), suggesting that risk or protection is dependent on specific sequence combinations between promoter and primary gene transcript. Such different combinations may be reflected in the different LD values observed between SNP1 and other SNPs in controls *vs* patients. However, associations with BPD were also found in other configurations. Interestingly, the region mapping between introns 7 and 8, that is, the one containing SNPs 4 and 5, which have been often reported in the literature associated with suicide and violent behavior, showed the least significant associations.

Although we report robust associations, our conclusions might be limited by several factors. First, we cannot exclude that differences in observed LD values between groups are related to population stratification. In particular, as noted in the Materials and methods, our BPD patients also had one to several diagnoses on Axis I. Also, the high LD levels observed between SNPs could be a source of redundant information in our haplotype analyses. On the other hand, it should be mentioned that the Bonferroni correction used throughout this study is very conservative, particularly in our case where measurements are not independent since the SNPs are in high LD. Finally, given the limited number of subjects analyzed in this study, our results will have to be reproduced in replica populations to be confirmed.

TPH has recently become the focus of renewed attention, as a novel isoform named TPH-2 has been discovered (Walther and Bader, 2003). While the two genes have little overall sequence homology, on the amino-acid level the proteins share about 70% identities (Walther and Bader, 2003). TPH-2 is the predominant isoform expressed in the brain stem, the major locus of 5-HT-producing neurons (Zill *et al*, 2004a, b). However, TPH-1 and TPH-2 mRNA are expressed in nearly equal amounts in human brain regions such as frontal cortex, thalamus, hippocampus, hypothalamus, and amygdale (Zill *et al*, 2004a, b). There is extensive evidence for the presence of 5-HT nerve terminals and receptors in neuroendocrine regions such as the hippocampus and the hypothalamus (Chaouloff, 1993; Graeff, 1993). Also, 5-HT turnover is particularly active in cortical and limbic areas involved in emotional aspects of behavior (Whitaker-Azmitia *et al*, 1990; Westenberg, 1996). A *TPH-2* haplotype analysis has recently revealed an association of the *TPH-2* gene with suicidal behavior (Zill *et al*, 2004a). Given the close interplay between the two TPH isoforms in key brain areas related to mood and impulse control, an understanding of their relative role in pathogenic events leading to depression will require further studies on a molecular level.

The statistical significance obtained from our haplotype analysis differs greatly from that usually obtained from single locus associations including those reported here. It appears that single SNP analysis fails to capture the underlying, specific gene variant association with disease phenotype that can instead be highlighted by haplotype analysis. The potential outcome of haplotype analysis in clinical studies has been already predicted (Judson *et al*, 2000; Clark, 2004). In addition to an improved genotype–phenotype association, our studies provide a basis for a complete molecular characterization of *TPH-1* risk variants. This in turn will facilitate the functional characterization of a gene variant's functional features, with a potentially immediate translation into disease pathogenesis studies.

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