Panic disorder and serotonergic genes (*SLC6A4*, *HTR1A* and *HTR2A*): Association and interaction with childhood trauma and parenting

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ABSTRACT

Objective: The aim of this study is to evaluate the association between *HTR1A*, *HTR2A* and the 5-HTTLPR in panic disorder (PD) patients and controls. In addition, this study also aims to evaluate the interaction between these genes and two environmental factors previously associated with PD: childhood trauma and parental bonding.

Methods: This is a case–control candidate gene association study (107 PD patients and 125 controls). Genes were analyzed using a gene-based test in PLINK followed by single marker association tests and haplotype test only for genes that reached experiment-wide significance in the gene-based test in order to minimize multiple testing. Logistic regression was used to test the relationships between genotype in the additive model, trauma, optimal paternal parenting and optimal maternal parenting and their interactions.

Results: Only *HTR1A* was associated with PD in gene-based test after correction for multiple tests ($p_{corrected} = 0.027$) and one *HTR1A* haplotype comprising four SNPs was associated with PD ($p_{corrected} = 0.032$). In the interaction analysis, no significant gene–environment interaction was found with the genes evaluated.

Conclusion: This study reinforces the association between *HTR1A* and PD. No major evidence of gene–environment interaction in PD with parenting or trauma was found. Further studies are necessary in order to confirm these findings.

Panic disorder (PD) is a familial and heritable phenotype [42]. However, efforts to identify the specific genes involved have had mixed results [3,8,24]. Association studies have focused on the role played by the genes encoding the elements of specific neurotransmitter system, including receptors, transporters and metabolizing enzyme of the serotonergic, noradrenergic or colecystokininergic systems [14]. Serotonergic neurotransmission pathways involve potential candidate genes since experimental studies have shown that lowering serotonin levels by tryptophan depletion augments panic response in PD patients [25]. In addition, medications increasing the synaptic availability such as serotonin re-uptake inhibitors are effective in the treatment of PD [25].

Previous studies have found a significant association between the short form (s allele) of the serotonin transporter gene (*SLC6A4*) promoter polymorphism (5-HTTLPR) and anxiety traits in healthy volunteers [40]. In a meta-analysis [3], there was no association found between PD and *5-HTTLPR*. However, a recently detected genetic polymorphism (16A allele) [30] in the l allele of *5-HTTLPR* has been linked to a lower expression of the serotonin transporter [17,21] and no study had previously evaluated this polymorphism in association studies of panic disorder. Pre-clinical and clinical studies have suggested that the serotonin receptor 1A gene (*HTR1A*) might play a critical role in PD [16] and a functional promoter polymorphism in the *HTR1A* C1019G (rs6295) has been reported to be associated to agoraphobia and PD [37]. The serotonin receptor 2 gene (*HTR2A*) has also been implicated in PD etiology since drugs that act on this serotonin receptor are anxiolytic [6]. These results warrant further investigation of the role of genes of the serotonin pathway in PD.

While genes influence the risk of PD, environmental risk factors such as childhood experiences play an equally important role [11]. For instance, childhood sexual abuse elevates the risk

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for adult generalized anxiety disorder and PD [11,20]. Some authors have also suggested that the relationship of a child with the primary caregiver can play an important role in the development of psychopathology [10,22,35]. Previous studies have reported that PD is associated with lower parental care and higher overprotection scores when compared to healthy controls [10,31,44].

Diathesis-stress theories of depression and anxiety suggest that individuals' sensitivity to stressful events depends on their genetic makeup. Heritability estimates for anxiety symptoms are higher among individuals who have reported stressful life events as compared to those without stress, suggesting environmental moderation of genetic effects [41]. Association studies attempt to identify both environmental and genetic risk factors as well as their interaction to test if a specific genotype moderates the effect of exposure to an environmental risk factor [26]. Serotonergic genes have been implicated in a gene–environment studies of depression [5,19] and anxiety [12]. However, no study to date has investigated gene–environment interactions in PD.

The aim of this study is to evaluate the association between polymorphisms in *HTR1A*, *HTR2A*, 5-HTTLPR and PD. Additionally, we investigate the interaction between polymorphisms in these genes and two environmental factors previously associated with PD: childhood trauma and parental bonding.

PD patients with or without agoraphobia were recruited from the Anxiety Disorder Outpatient Clinic at the Hospital de Clínicas de Porto Alegre (HCPA) after informed consent. Subjects comprised of 109 PD patients and 127 controls who were employees from HCPA and were recruited between June 2006 and April 2007. Patients and controls were screened by a psychiatrist to evaluate any psychiatric disorders. All subjects were at least 18 years old and ethnically European-Brazilian. Patients with comorbidities common to PD were eligible, provided that the symptoms were not clinically more severe than the PD symptoms. Blood was collected from participants for DNA extraction after written informed consent. Institutional review board approval was obtained from the ethics committee of the Hospital de Clínicas de Porto Alegre (Number 04-272) and the study is in accordance with the Declaration of Helsinki.

All subjects were evaluated by a clinical interview and the Mini International Neuropsychiatry Interview (MINI) – Brazilian version [1]. A semi-structured interview was used to assess sociodemographic data and clinical history.

Childhood trauma was evaluated by the Childhood Trauma Questionnaire (CTQ) – Brazilian version [13], which evaluates five subscales: childhood emotional, physical and sexual abuse and childhood physical and emotional neglect. Previous studies indicate that the CTQ scores are stable and subscales demonstrated good internal consistency and convergent validity. Since all subscales were associated with PD (Mann–Whitney U, $p < 0.01$) and were intercorrelated ($r > 0.4$), CTQ was only assessed as the global index to avoid multiple comparisons [38].

Parenting was assessed by the Parental Bonding Inventory (PBI) – Brazilian version [15,39]. The original PBI was intended to measure the perceived parental rearing styles of overprotection and warmth as remembered by the respondents during their first 16 years of life. It consists of 25 items to be assessed separately for mother and father. The long-term stability of the PBI has been demonstrated in a 20-year cohort study. Both low warmth and high overprotection were associated with PD (Mann–Whitney U, $p < 0.01$), but no association was found with authoritarianism in either the father or mother with PD. In order to minimize multiple comparisons, we generated a combined variable for all the scales related to each parent, creating a 2×2 table considering warmth (low and high) and overprotection (low and high) and then using only one quadrant as the ideal parenting subtype named "optimal

father" and "optimal mother" characterized by high warmth and low overprotection scores.

For 5-HTTLPR, we examined the variable length polymorphism in the promoter including the embedded SNP (rs25531). For HTR2A, we included two putative functional SNPs: rs6313 (located in a coding exon) and rs6311 (located in the promoter). For HTR1A, HapMap (CEU, data release 24) SNPs were selected within the gene and 10 kb flanking region to each side, resulting in a span of 20 kb. The Tagger program [7] (http://www.broad.mit.edu/mpg/tagger/) was used to identify tag SNPs with a minor allele frequency of greater than 5% and minimum r^2 of 0.8. Six markers for HTR1A were selected: rs12653018, rs4521432, rs6449693, rs6294, rs7448024 and rs13361335 using pairwise marker tagging. Additionally, one functional SNPs (rs6295) previously evaluated in PD sample [37] were also included.

A total of 1 μg genomic DNA (diluted in 1× TE buffer at 50 ng/μl) from each subject was transferred to 96-well plates. Quantification of DNA was determined by PicoGreen® (Molecular Probes, OR, USA).

Genotyping of HTR1A and HTR2A was performed as previously described [43]. Genotyping of the 5-HTTLPR was performed using the following protocol: Genomic DNA (1.4 ng) was amplified in a 7 μl reaction using KlenTaq DNA Polymerase (0.2 U), the proprietary KlenTaq Buffer (1×), dNTPs (200 μM each), 5% glycerol, Betaine (1 M) and the marker specific primers (0.2 μM). Amplification was performed with 13 cycles of denaturation for 30 s at 93 °C, annealing for 30 s beginning at 61.5 °C and dropped 0.5 °C every cycle and primer extension at 72 °C for 30 s, followed by 37 cycles of denaturation for 30 s at 93 °C, annealing for 30 s at 55 °C and primer extension at 72 °C for 30; 72 °C for 1 h. The amplified product (1 μl) was combined with size standard (LIZ-250) before being analyzed on an ABI-3730. The long allele appears as a product of about 412 while the short allele shows a band at about 370. The genotype of the SNP embedded in the 5-HTTLPR (rs25531) was assayed by digesting the PCR product with the restriction enzyme MspI [17]. This 10 μl reaction contains 2 μl of the PCR product, 1 μl of 10× restriction buffer (New England Biolabs), 1 μl of MspI enzyme and 6 μl of water. The reaction was incubated at 37 °C for 1 h. The digested product (1 μl) was then combined with size standard (LIZ-250) before being analyzed on an ABI-3730. Post-digestion, the long allele with the A SNP allele appears as a product of about 320, the G SNP allele is indicated by the presence of a band at 148, while the short allele shows a band at about 277. The final genotype was determined from the information from the analysis of both the digested and undigested PCR product.

Markers were retained only if they met the following quality control criteria: (1) >90% genotype call rate; (2) minor allele frequency (MAF) >5%; (3) Hardy–Weinberg equilibrium $p > 0.001$. Three duplicates were assessed in the experiment and 100% concordance in genotypes was seen. After applying these quality control filters, 8 markers were retained for the association analysis (rs4521432, rs6449693, rs13361335, rs6295, rs6313, rs6311, *5-HTTLPR* biallelic and triallelic). Two SNP (rs6294 and rs12653018) did not meet our thresholds for minor allele frequency, and one SNP (rs7448024) was out of HWE.

Four individuals with genotyping call rates less than 90% were excluded resulting in final dataset of 232 (107 cases and 125 controls) subjects to be included in the analysis.

Normal distribution and sphericity of phenotypes were assessed using Kolmogorov–Smirnov test and Levene's test. The majority of CTQ and PBI scales are not normally distributed and so the CTQ and PBI scores were dichotomized using ROC curves to maximize the accuracy of the cut-off to predict presence of PD in adulthood for risk factors, or predict absence of PD in adulthood for protective factors. The Youden's index J was used to define the optimal cut-point for the continuous variables and area under the curve (AUC) was

Table 1
Set-based test for the association between polymorphisms (PMs) and panic disorder (PD $n = 107$, Controls $n = 125$).

Set	PMs in the set	PMs	T-Value	Empirical p-value 0	Empirical p-value 1	Empirical p-value 2
HTR1A	P1	rs6449693	7.495	0.0159	0.0203	0.0555
	P2	rs4521432	7.226	0.0103	0.0137	0.0383
	P3	rs6295	7.059	0.0089	0.0111	**0.0303**
HTR2A	P1	rs6311	0.335	0.6337	0.6493	0.9673
	P2	rs6313	0.238	0.6415	0.6493	0.9677
5-HTTLPR	P1	Biallelic	0.318	0.5975	0.5975	0.9597
5-HTTLPR	P1	Triallelic	0.225	0.6469	0.6469	0.9717

A multi-marker set-based test implemented in PLINK was used for the analysis of all genes. Sets that have an experiment-wide association p-value ($p < 0.05$) are shown in bold. In each set, P1 is the best PMs associated with the outcome; P2 is the second one and P3 is the third one. Maximum computed permutations were 10,000. Empirical p-value p 0: p-value for average chi-square without correction; empirical p-value p 1: p-value corrected for all tests within this set/gene; empirical p-value p 2: p-value corrected for all tests in all sets. *Abbreviations*: PMs, polymorphisms; *HTR1A*, serotonin receptor 1A; HTR2A, serotonin receptor 2A; 5-HTTLPR, serotonin transporter gene promoter polymorphism.

used to test significance. The best cut-point for CTQ Global Score was ≥ 41 (AUC = 0.695; $p < 0.001$). The best cut-points for PBI scales were: ≥ 6 for maternal overprotection (AUC = 0.621; $p = 0.002$) and ≥ 4 for paternal overprotection (AUC = 0.631; $p = 0.001$); and ≥ 16 for maternal warmth (AUC = 0.615; $p = 0.003$) and ≥ 13 for paternal warmth (AUC = 0.600; $p = 0.001$). This procedure has been used before in a previous study [39].

For an initial analysis, we used a set-based test for each gene implemented in PLINK [33] (http://pngu.mgh.harvard.edu/purcell/plink/) followed by single marker association tests. The set-based test is based on calculating the average test statistic for the best three SNPs per gene region, and evaluating the significance of these set-statistics by permutations ($m = 10,000$).

Haplotype analysis was performed for genes that reached an experiment-wide significance. Haplotypes for *HTR1A* was defined using Haploview 4.2 [2]. The haplotype test performed is an omnibus test to evaluate the joint effect of all haplotypes observed (minor allele frequencies [MAF] > .01). For haplotypes that met the significance criteria in the omnibus test, we performed haplotype-specific tests of each haplotype versus all others corrected with permutation ($n = 10,000$ permutations).

Logistic regression techniques in PLINK were used to model the relationships between genotype (using an additive model) and trauma, optimal paternal parenting and optimal maternal parenting, independently. For each polymorphism, the model included: genotype, one of the three environmental factors and their interaction term (genotype × environment).

At each stage in the analysis, we adjusted the p-values to incorporate correction for multiple hypothesis testing. For single marker association analysis, permutation ($n = 10,000$ permutations) was used to correct for multiple testing yielding both gene-wise (p_1) and experiment-wise (p_2) significance values. Haplotype analysis was corrected with permutation ($n = 10,000$ permutations) using Haploview 4.2. The interaction analysis was corrected using Bonferroni's correction, accounting for the eight polymorphisms evaluated. All tests were two-tailed.

The final dataset comprised 107 patients (76.6% woman, mean age 39.94, SD 10.17 years) and 125 controls (70.4% woman, mean age 36.93, SD 9.78 years). The mean age of PD onset was 32 (SD = 10.38) years, and mean PD duration of 7.94 years (SD = 8.45). Major PD comorbidities included agoraphobia (89.7%), major depression (29%), generalized anxiety disorder (37.4%), social anxiety disorder (17.8%), and dysthymia (15.9%).

Table 1 shows the results for the SNP association analysis with PD. The results show that two markers in *HTR1A* remain significant after experiment-wise correction for set-based tests. Table 2 shows results for the single marker association and haplotype association analyses with PD for the HTR1A markers.

In the whole sample, rs4521432, rs6449693, rs6295 and rs13361335 were in strong LD (supplementary results, Fig. I). In the haplotype analysis (omnibus test $p = 0.032$), Hap2 (Table 2) was significantly associated with PD (asymptotic p-value = 0.008), whereas Hap4 was protective (asymptotic p-value = 0.004) and remained significant after permutation.

Table 2
HTR1A single marker association and haplotype association with panic disorder (107 cases and 125 controls).

	rs4521432	rs6449693	rs6295	rs13361335	Frequency in PD	Frequency in Controls	χ^2 (df = 1)	Asymptotic p-value	Corrected p-value
Hap1	C	A	C	G	0.12	0.11	0.11	0.736	>0.999
Hap2	T	G	G	T	0.55	0.42	6.97	0.008	0.032
Hap3	C	A	G	T	0.01	0.02	0.03	0.871	>0.99
Hap4	C	A	C	T	0.32	0.45	8.29	0.004	0.009
				Ominbus .032					
Role	Downstream	Downstream	Promoter	Promoter					
HWE	0.695	0.794	0.794	>0.999					
Allele change	C/T	A/G	C/G	G/T					
Minor allele	T	G	G	G					
Frequency in PD	0.55	0.55	0.56	0.12					
Frequency in Controls	0.42	0.42	0.44	0.11					
χ^2 (df = 1)	6.96	7.49	6.72	0.09					
Asymptotic p-value	**0.008**	**0.006**	**0.009**	0.764					
Empirical p-value 1	**0.024**	**0.015**	**0.026**	0.969					
Empirical p-value 2	0.053	**0.041**	0.066	0.999					
OR (95% CI)	1.64 (1.13–2.37)	1.67 (1.16–2.41)	1.62 (1.12–2.35)	1.09 (0.61–1.94)					

Abbreviations: PD, panic disorder; *HTR1A*, serotonin receptor 1A; SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium p-value; OR, odds ratio; χ^2, chi-square; df, degrees of freedom; 95% CI, confidence interval of 95%. Bold means $p < 0.05$. Asymptotic p-value: p-value for chi-square without correction; corrected p-value: p-value corrected for with 10,000 permutations.

The interaction of trauma and 5-HTTLPR was nominally associated with PD, but did not remain significant after Bonferroni correction ($p = 0.32$). There were no significant results from analyses of interaction between any SNPs and optimal maternal or paternal parenting, or trauma in childhood and PD in adulthood (supplementary results, Table 3).

As reported in previous studies [18,36], we observed evidence of association between variants in HTR1A and PD. In this study, we also observed an association between PD and a specific HTR1A haplotype. In addition, we found no gene–environment interaction between childhood experiences and 5-HTTLPR, HTR1A and HTR2A and PD.

Pre-clinical [32] and clinical studies have implicated HTR1A in the pathogenesis of PD [4,23]. Studies in HTR1A knockout mice have demonstrated increased anxiety-like behavior in several tests [9,32,34]. In addition, PD has been associated with a reduction in both pre-synaptic and post-synaptic HTR1A receptors, as assessed by positron emission tomography [28]. Previous studies evaluating a functional SNP (rs6295) in PD have found inconsistent results [24]. In our study, we saw associations of several SNPs in HTR1A with PD.

Similar inconsistent results have been published regarding associations of HTR2A and PD [24]. The 5-HTTLPR short allele has been associated with anxiety traits in healthy volunteers [40]. Regarding PD, no association was found between this polymorphism in a meta-analysis [3] or in our study. To our knowledge, this is the first study that has evaluated the triallelic form [17] of 5-HTTLPR and PD, and no significant results were found for either main effects or gene–environment interactions.

We did not find any evidence of gene–environment interaction with 5-HTTLPR, HTR1A or HTR2A using a composite trauma index or indices of optimal maternal or paternal parenting, though again power was limited for this analysis. Gene–environmental interaction with trauma and 5-HTTLPR has been reported for depression by different authors, but a recent meta-analysis did not support this interaction [27,29]. Our findings regarding PD are in agreement with this lack of association reported for depression.

Our results should be interpreted in light of several limitations. Most importantly, the small sample size means that our analyses may be subject to Type II error. The possibility of confounding by population stratification exists in our study, though our sample was restricted to self-identified Caucasian individuals as an attempt to reduce these possible biases. A recent study has shown that in the south of Brazil (where this sample is from), few inconsistencies were seen between self-reported ethnicity and genetic data [45]. Additionally, the assessment of childhood maltreatment and parental bonding was retrospective and done at the same time as the phenotypic evaluation.

In sum, these data support an association between HTR1A and PD, consistent with previous studies. This study is among the first to evaluate the effect of gene–environment interactions in PD, but we could not find any interaction between childhood experiences, serotonergic genes and adult PD. Additional studies with larger sample sizes are required to fully evaluate the potential for gene–environment interaction.

Conflict of interest

CB is on the speaker bureau of Eli Lilly Brazil. GGM is on the speaker bureau of Eli Lilly and Roche Brazil. JWS has consulted to Eli Lilly, received honoraria from Hoffman-La Roche, Inc., Enterprise Analysis Corp., and MPM Capital, and has served on an advisory board for Roche Diagnostics Corporation. PM, GAS, EH, ACS and SL-S have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neulet.2010.08.042.

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