



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 ($p_{\text{corrected}} = 0.027$) and one *HTR1A* haplotype comprising four SNPs was associated with PD ($p_{\text{corrected}} = 0.032$). 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.

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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 orolecystokinergic 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 μ g genomic DNA (diluted in $1 \times$ TE buffer at 50 ng/ μ l) 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 μ l reaction using KlenTaq DNA Polymerase (0.2 U), the proprietary KlenTaq Buffer ($1 \times$), dNTPs (200 μ M each), 5% glycerol, Betaine (1 M) and the marker specific primers (0.2 μ M). 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 μ l) 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 μ l reaction contains 2 μ l of the PCR product, 1 μ l of $10 \times$ restriction buffer (New England Biolabs), 1 μ l of MspI enzyme and 6 μ l of water. The reaction was incubated at 37 °C for 1 h. The digested product (1 μ l) 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 SNPs (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

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