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ARTICLE

Serotonin transporter 5HTTLPR polymorphism and affective disorders: no evidence of association in a large European multicenter study

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The available data from preclinical and pharmacological studies on the role of the serotonin transporter (5-HTT) support the hypothesis that a dysfunction in brain serotonergic system activity contributes to the vulnerability to affective disorders (AD). 5-HTT is the major site of serotonin reuptake into the presynaptic neuron, and it has been shown that the polymorphic repeat polymorphism in the 5-HTT promotor region (5-HTTLPR) may affect gene-transcription activity. 5-HTT maps to chromosome 17 at position 17q11.17–q12, and the 5-HTTLPR polymorphisms have been extensively investigated in AD with conflicting results. The present study tested the genetic contribution of the 5-HTTLPR polymorphism in a large European multicenter case–control sample, including 539 unipolar (UPAD), 572 bipolar patients (BPAD), and 821 controls (C). Our European collaboration has led to efforts to optimize a methodology that attenuates some of the major limitations of the case–control association approach. No association was found with primary psychiatric diagnosis (UPAD and BPAD) and with phenotypic traits (family history of AD, suicidal attempt, and presence of psychotic features). Our negative findings are not attributable to the lack of statistical power, and may contribute to clarify the role of 5-HTTLPR polymorphism in AD.
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Introduction

The serotonin (5-hydroxytryptamine) transporter (5-HTT), responsible for serotonin reuptake into presynaptic neurons, regulates the concentration of serotonin in the synaptic cleft and constitutes a key protein in the complex serotonergic pathway. It has been hypothesized that serotonin is implicated in the pathophysiology of several psychiatric disorders such as affective disorders (AD), autism, obsessive compulsive, and anxiety disorders.[1] Moreover, 5-HTT has been reported to have a role in such behaviors as sleep, appetite, aggression, memory, nutrient intake, impulsivity, and personality traits[2] (neuroticism, harm avoidance). At a pharmacological level, 5-HTT is the site of binding for tricyclic antidepressants and serotonin reuptake inhibitors – SSRI, thus reducing serotonin reuptake. The 5-HTT gene maps to chromosome 17 at position 17q11.17–q12, and is a strong candidate gene for AD.[3]

Two 5-HTT polymorphisms have been extensively investigated in AD. The first polymorphism is a variable number of tandem repeats (VNTR) in intron 2, which has been reported to act as a transcriptional regulatory element of the 5-HTT in mice and human.[4–6] The second VNTR polymorphism is composed of 16 repeat elements, and consists of a 44-bp insertion or deletion (involving repeat elements 6–8), located exactly at the 5′-flanking regulatory region of the serotonin transporter gene on chromosome 17q11.2 (approximately 1 kb upstream of the transcription initiation site), that is, serotonergic transporter-linked polymorphic region (5-HTTLPR). There are two common variants, designated 'long' (or 'L') and 'short' (or 'S').[7] Studies reported functional repercussions such that the L and S alleles have different transcriptional efficiencies. The L allele has a higher transcriptional activity *in vitro* and in lymphoblastoid cell lines, compared to SS homozygotes.[7,8] Consequently, the rate of removal of serotonin from the synaptic cleft varies according to the distribution of alleles, indicating that the number of repetitive sequences in the 5-HTTLPR polymorphism may have a role in the transcriptional activity. These reports have generated considerable interest, and a very large number of investigations have been conducted on these two 5-HTT polymorphisms in AD. However, conflicting results from association studies do not clarify the role of 5-HTT in phenotypes such as unipolar (UPAD) or bipolar disorders (BPAD) (see the review of Bellivier *et al*,[9] which summarized results of these studies). Additionally, negative linkage studies have also been reported in multiplex families.[10–12] Lack of statistical power, clinical heterogeneity, genetic heterogeneity, and ethnogeographic stratification as source of bias in association studies may account for the conflicting results. The genetic basis of AD is complex, involving interaction between genetic and environment factors, and most likely involves the interaction of several genes of minor effect. One cannot assume that the presence of an allele is either necessary or sufficient to 'cause' the phenotype. The power of association analysis to detect genetic contributions to complex diseases can be greater than linkage studies,[13,14] when the sample size gives sufficient power to detect genes of minor effect and when patients and controls are recruited using strict and standardized inclusion criteria, and are well matched for geographical origin. To further clarify the involvement of 5-HTT in AD, we carried out a case–control association study in a large European multicenter sample, using the 5-HTTLPR functional polymorphism. A total of 1932 subjects were selected with DNA and clinical data: 539 unipolar (UPAD), 572 bipolar patients (BPAD), and 821 control subjects (C). To our knowledge, our sample size constitutes one of the largest samples examined for the 5-HTTLPR polymorphism in AD (Table 1).

Methods

Subjects

The present sample was recruited within the Biomedical European (BIOMED 2) European Collaborative Study on Molecular Genetics in Affective disorders, Contract No: BMH4-CT-97-2307. This network was established within the framework of the European Commission. The objectives and detailed methodology of the project were described previously.[15] A total of eight clinical centers participated in the present study (Edinburgh, Brussels, Sofia, Zagreb, Bonn, Jerusalem, Milan, and Umeå; see Table 1). All subjects were interviewed using standard diagnostic interviews, such as the Schedule for Affective Disorders and Schizophrenia-Lifetime Version (SADS-LA)[16] and the Schedule for Clinical Assessment of Neuropsychia-

Table 1 Contribution of each center to the recruitment of patient–control samples

Center	*BPAD*	*UPAD*	*Controls*	*Total*
Edinburgh	140	127	42	309
Brussels	66	65	95	226
Sofia	47	86	104	237
Zagreb	42	88	116	246
Bonn	40	40	202	282
Jerusalem	67	84	91	242
Milan	49	82	93	224
Umeå	88	—	78	166
Total	539	572	821	1932

try (SCAN).[17] One of the two diagnostic interviews was used for all patients and all controls recruited for the project. The choice of the diagnostic interviews was based on the research experience within individual research teams. Data published by the European Science Foundation (ESF) showed good concordance between the two instruments.[18] A total of 1932 subjects were selected with DNA and clinical data: 539 UPAD subjects including 188 males, 349 females, and two missing data, 572 BPAD subjects including 242 males, 328 females, and two missing data, and 821 controls including 363 males, 441 females, and 17 missing data. The age distribution was 53.8 years for UPAD, 49.7 years for BPAD, and 47.3 years for controls. Patients met the diagnosis of BPAD and UPAD, according to RDC, DSM-III-R, and DSM-IV classification systems. To test alternative approaches based on phenotypic traits, we selected clinical subgroups, characterized by a positive family history of AD in first-degree relatives ($n=94$ BPAD; $n=71$ UPAD), antecedents of suicidal attempt ($n=104$ BPAD, $n=63$ UPAD), and presence of psychotic features (delusion or hallucination, $n=103$ BPAD, $n=17$ UPAD). Family data were assessed using the Family History RDC (FH-RDC)[19] instrument. When available, family data were also collected from relatives. Positive family history was defined as having at least one first-degree relative affected with BPAD or UPAD. Subjects for whom clinical information to categorize them into those subgroups were not available were not included in these analyses. In the control group, subjects with a positive personal or familial history of psychiatric disorder were excluded. The study was approved by local research ethics committees and informed consent was obtained from patients and controls.

Genotyping

PCR was carried out in a 20–50 μl reaction volume containing 20–100 ng of genomic DNA and 10 pmol of each primer. The primers used in this study were as described by Heils[7] *et al* or Cook[20] *et al*. In all, 200 μM of dATP, dTTP, and dCTP were used together with 100 μM dGTP and 100 μM 7-deaza-dGTP, 1 U of Taq DNA polymerase with the associated buffer containing 1.5 mM Mg2Cl and 5% DMSO. Following heat denaturation of the samples (5 min at 95°C), 35–40 cycles were carried out consisting of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by a final extension step of 5 min at 72°C. PCR products were resolved on a 3% agarose gel or on a 10% polyacrylamide gel. Bands were visualized by ethidium bromide staining under UV illumination.

Statistical analysis

The Hardy–Weinberg equilibrium (HWE) was tested separately in the three groups (UPAD, BPAD, and controls), by using the exact HW test and the GENEPOP, 3.d. program-updated version of GENEPOP.[21] No significant deviation from HWE was observed in any of the three groups. Genotypes' distribution in controls (LL 33%, LS 47.1%, SS 19.6%) was comparable to that reported by Lesch *et al*[8] in a European population. The empirical statistical power of our two samples (UPAD compared to controls and BPAD compared to controls), based on simulations (odd ratios comprised between 1, 9, and 4 (effect size comprised between 0.16 and 0.3) according to the findings in the literature, and allelic frequencies in controls comprised between 40 and 50%) was more than 90%. It was calculated with program G power.[22] Matching in the design was utilized to control for potential confounding factor, such as ethnogeographical origin, a complex nominal variable which represents a wide and indefinable range of environmental and genetic factors difficult to quantify and thus to control by other means. Matching cases and controls in the design were used. Since there were more controls than cases in each center, except Edinburgh, each case was individually matched for ethnogeographical origin to one or several controls within each center (each set was composed of one case and *N* controls, *N* varying from a set to another, from 1 to 6). In Edinburgh center, where more cases than controls were ascertained, each control was matched to one or two cases. The number of controls/cases per case/control was allowed to vary from set to set. The same controls were used to be matched with UPAD and BPAD cases. Owing to the matched design, Conditional Logistic Regression for matched sets[23] was used to assess the association between dependant variables (diagnosis *vs* control) and predictors (genotypes/alleles), and to derive odds ratios and 95% confidence intervals after adjustment for potential confounding factors. Estimated coefficients were provided for each of the covariates. We conducted the conditional logistic regression with SPSS, using the coxreg procedure to fit a conditional logit model. The overall χ^2 (Wald) is provided for each analysis. However, the limitation of matching is the inability to evaluate the effect of matching factor on risk of the outcome. To evaluate the possible modifying effect of center and gender, a stratified analysis was performed (match ignoring) and odds ratios (OR) and confidence intervals (95% CI) were calculated. The Breslow–Day test[23] was applied to test the homogeneity of the stratum-specific estimates (center and gender for strata and presence of genotype S–S as exposition). To evaluate the possible confounding effect of gender, the Mantel–Haenszel test[24] was applied. Bonferroni correction for multiple tests was applied, when necessary (for four tests: comparisons (patients/controls; patients with family history of AD/controls; patients with suicidal attempt/controls and patients with psychotic features/controls).

Results

Polymorphism HTTLPR and BPAD

The initial focus of our analysis was to detect differences between BPAD and C subjects, in the distribution of

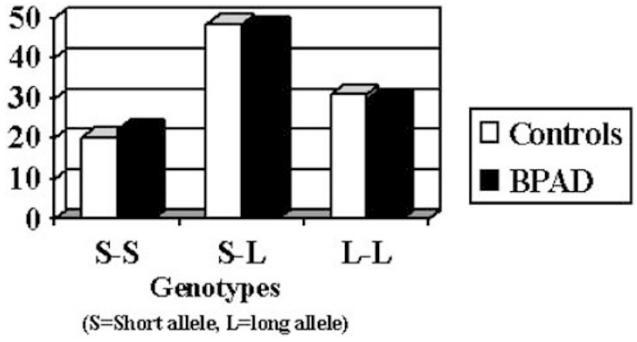

Figure 1 Genotypic distribution (%) between BPAD and controls for 5-HTTLPR.

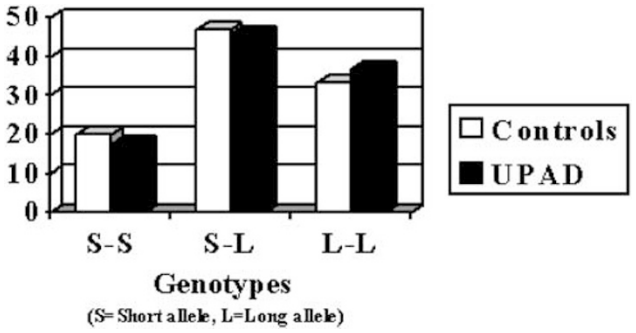

Figure 2 Genotypic distribution (%) between LPAD and controls for 5-HTTLPR.

genotypes and alleles. No significant differences emerged ($\chi^2=0.1$, df = 2, $P=0.95$ for genotypes and $\chi^2=0.07$, df = 1, $P=0.8$ for alleles distributions – see Figure 1). Subgroup examinations did not show significant differences in genotype and allelic distributions ($\chi^2=2.9$, df = 2, $P=0.23$ and $\chi^2=0.98$, df = 1, $P=0.32$ when considering the group of BPAD with family history of AD compared to controls; $\chi^2=2.9$, df = 2, $P=0.23$ and $\chi^2=0.99$, df = 1, $P=0.31$, the group of BPAD with a history of suicide attempt compared to controls; $\chi^2=3.9$, df = 2, $P=0.14$ and $\chi^2=0.43$, df = 1, $P=0.5$, when considering the group of BPAD with psychotic features compared to controls).

Stratification tests excluded confounding and modifier effects of gender and center (see Table 2).

Polymorphism HTTLPR and UPAD

No significant association was detected comparing genotype and allele distributions between UPAD patients and control groups ($\chi^2=0.62$, df = 2, $P=0.73$ and $\chi^2=0.46$, df = 1, $P=0.49$, see Figure 2) and subgroups (UPAD with family history of AD/controls $\chi^2=7.1$, df = 2, $P=0.03$ (NS (nonsignificant) after Bonferroni correction for four tests: $P=0.12$; $\chi^2=1$, df = 1, $P=0.31$; UPAD with history of suicidal attempt /controls $\chi^2=4.6$, df = 2, $P=0.09$; $\chi^2=0.79$, df = 1, $P=0.37$; UPAD with psychotic features/ controls $\chi^2=4.7$, df = 2, $P=0.09$; $\chi^2=0.00$, df = 1, $P=0.98$).

As for the previous analysis, no confounding or modifier effects emerged for gender and center (see Table 2).

Additional analyses (not shown) performed to test the combined BPAD and UPAD groups, showed no association, when we considered primary diagnosis of AD and phenotypic traits such as suicidal attempt, psychotic features and family history of AD.

Finally, for each group of patients (UPAD and BPAD), we tested the effect of age and age at onset of episode. No age effects emerged on frequencies of genotypes (results not shown).

Discussion

In this multicenter European association study, we collected one of the largest sample sizes of BPAD, UPAD and controls, using strict and standardized inclusion criteria for patients and controls. Our European collaboration has led to efforts to optimize a methodology that reduces some of the most severe limitations of the case–control association approach (such as stratification bias). In particular, we carefully matched patients and controls for geographical origin and used an appropriate statistical method for matched samples. No association was found

Table 2 Stratification tests: Mantel–Haenszel tests (MH) and Breslow–Day test (BD) – dichotomic variable: genotype S–S

	Gender: MH test	OR_{MH}	OR_{Crude}	*Gender: BD test*	*Center: BD test*
BPAD groups compared to controls					
BP	$\chi^2=0.46$, df = 1, $P=0.49$	0.89	0.89	$\chi^2=1.4$, df = 1, $P=0.23$	$\chi^2=8.5$, df = 6, $P=0.2$
BP+family history of AD	$\chi^2=2.73$, df = 1, $P=0.09$	0.6	0.6	$\chi^2=0.06$, df = 1, $P=0.8$	$\chi^2=1.8$, df = 5, $P=0.85$
BP+history of suicide attempt	$\chi^2=1.51$, df = 1, $P=0.21$	0.71	0.73	$\chi^2=2.5$, df = 1, $P=0.11$	$\chi^2=2.5$, df = 7, $P=0.7$
BP+psychotic features	$\chi^2=3.3$, df = 1, $P=0.07$	0.63	0.64	$\chi^2=0.25$, df = 1, p = 0.87	$\chi^2=2.8$, df = 3, $P=0.4$
UPAD groups compared to controls					
UPAD	$\chi^2=0.81$, df = 1, $P=0.36$	1.15	1.17	$\chi^2=1.2$, df = 1, $P=0.27$	$\chi^2=4.03$, df = 7, $P=0.7$
UPAD+family history of AD	$\chi^2=0.005$, df = 1, $P=0.94$	1.03	1.03	$\chi^2=0.66$, df = 1, $P=0.8$	$\chi^2=2.9$, df = 4, $P=0.57$
UPAD+history of suicide attempt	$\chi^2=2,44$, df = 1, $P=0.11$	0.58	0.55	$\chi^2=0.17$, df = 1, $P=0.8$	$\chi^2=1.8$, df = 4, $P=0.77$
UPAD+psychotic features	$\chi^2=0.14$, df = 1, $P=0.7$	0.7	0.9	$\chi^2=2.4$, df = 1, $P=0.12$	$\chi^2=3.16$, df = 2, $P=0.2$

between the 5-HTTLPR polymorphism and groups of BPAD and UPAD. The very high statistical power and the exclusion of a center effect render our results reliable. For BPAD, our negative results are comparable to most previous independent reports[25–34] Only four studies reported an association between the S allele of 5-HTTLPR and BPAD.[35–38] In the case of family-based studies, three independent studies reported negative findings when testing for preferential transmission of alleles (transmission disequilibrium test) in samples of trios,[39–41] and two family haplotype analyses showed positive findings.[42,43] However, sample sizes suggested that these studies lacked power (substantial risk of statistical Type II error). Our present report also supports the results of most investigations for UPAD, which also failed to detect association with the 5-HTTLPR polymorphism.[25,29,33,36,41,44] Only two findings were in favor of an association.[35,37] In addition, conflicting association results were reported when considering different phenotypic transonographical behavioral traits and subgroups of AD (seasonal AD, suicidal behavior, anxiety-related traits, personality dimensions, response to antidepressants, see for a review Bellivier *et al*[9]). In our studies, subgroup analyses (family history of AD, suicidal attempt and psychotic features) failed to show any association with the 5-HTTLPR polymorphism variants. Owing to our large sample size, it does not seem that our negative findings are due to lack of statistical power. A special effort was made to collect one of the most important databases for this candidate gene in AD. We cannot totally exclude some implication of 5-HTT in some AD phenotypic traits. It is possible that the polymorphism 5-HTTLPR may be close to another adjacent polymorphism involved in AD, without being in linkage disequilibrium. This situation can exist when the population studied is subdivided into small subpopulations carrying mutations descending from different ancestors and present on different haplotypes. When studying the global population, observable associations in subpopulations may become nonsignificant. Another possibility is that the mutation is old enough for the marker and the adjacent susceptibility locus to be in linkage equilibrium again. Data available in a study that compared allelic frequencies of 5-HTTLPR in populations from several geographic regions of the world reported two common alleles, showing that the polymorphism must have arisen early in the evolutionary history of humans.[45] However, allele frequencies vary considerably, as a probable consequence of random genetic drift. Above all, as demonstrated by Gelernter's report,[45] the large potential for population stratification in studies using this polymorphism may explain some conflicting results, in particular spurious positive findings. Moreover, we cannot exclude the presence of genetic heterogeneity due to population history.

In summary, we conclude that, in our very large European sample, no association was found with primary psychiatric diagnosis of BPAD and UPAD, and some related phenotypic traits. These results are in line with most previous negative reports. The pharmacogenetic study of therapeutic agents involved in the serotonergic pathway remains nevertheless a promising approach for the future.[9,46,47]

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